Indonesia International Institute for Life Sciences

ENRICHMENT PROGRAM REPORT

Investigating the Role of a Novel Gene in Modulating Polyploid Giant Cancer Cell Formation and Multidrug Resistance in Pancreatic Cancer

STUDY PROGRAM
Biomedicine

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INDONESIA INTERNATIONAL INSTITUTE FOR LIFE SCIENCES (i3L)
YEAR OF SUBMISSION

RESEARCH REPORT

INVESTIGATING THE ROLE OF A NOVEL GENE IN MODULATING POLYPLOID GIANT CANCER CELL FORMATION AND MULTIDRUG RESISTANCE IN PANCREATIC CANCER

By
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Submitted to

i3L – Indonesia International Institute for Life Sciences
School of Life Sciences

in partial fulfilment of the enrichment program for the Bachelor of
Biomedicine

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Jakarta, Indonesia
2024

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Cohort : 2021

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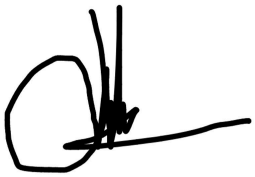

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Date: 23 January 2025

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ABSTRACT

Pancreatic cancer (PC), particularly pancreatic ductal adenocarcinoma (PDAC), is a highly lethal malignancy known for its poor prognosis. A major challenge in treating PDAC is multidrug resistance (MDR), which enables tumor cells to evade the effects of various chemotherapeutic agents. Additionally, the formation of polyploid giant cancer cells (PGCCs), characterized by polyploidy and reversible cell cycle arrest, complicates treatment further by promoting tumor recurrence. Understanding the role of Gene X is crucial, as it may provide insights into the molecular mechanisms underlying MDR and PGCCs in PDAC, potentially leading to novel therapeutic strategies. In this study, we found that the knockdown of Gene X led to significant cellular giantism, marked by enlarged cell sizes and irregular morphologies, indicating its critical role in maintaining normal cellular structure. Moreover, we found that although Gene X knockdown had a minor impact on sensitivity to Gemcitabine, it significantly increased resistance to Paclitaxel. This differential response highlights the potential role of Gene X in regulating pathways that are specific to the mechanisms of action of each drug, further emphasizing its relevance in the context of PDAC treatment challenges.

Keywords: Pancreatic Cancer, Polyaneuploidy Cancer Cell, PDAC, MDR

ACKNOWLEDGEMENTS

First and foremost, I would like to express my profound gratitude to the Lord above for granting me the opportunity to navigate the Enrichment Programme, a transformative journey filled with both challenges and triumphs. I extend my sincere appreciation to my field supervisor, Prof. Chen Hsin-Yi, and my i3L supervisor, Ms. Lidya Kristiani, S.Si., Ph.D., whose invaluable mentorship and unwavering support have significantly impacted my academic development. I am also thankful for the other lab members who have guided and taught me with remarkable patience and an open mind, fostering a collaborative environment that enriched my experience. Additionally, I wish to acknowledge my family and friends, whose their support and encouragement have been a source of strength during my time in Taiwan. Lastly, I would like to express my gratitude to Taipei Medical University (TMU) for hosting this programme and to the Indonesian International Institute for Life Sciences (i3L) for providing me with this incredible opportunity.

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LIST OF ABBREVIATIONS

ABC:	ATP-Binding Cassette
cDNA:	Complementary DNA
ddH2O:	Double-Distilled Water
DEPC:	Diethylpyrocarbonate
DMEM:	Dulbecco's Modified Eagle Medium
DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribonucleic Acid
FBS:	Fetal Bobine Serum
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
GFP:	Green Fluorescent Protein
KD:	Knockdown
MDR:	Multidrug Resistance
mRNA:	Messenger RNA
MTT:	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
NTC:	Non-Template Control
PGCC:	Polyploid Giant Cancer Cell
PBS:	Phosphate-Buffered Saline
PC:	Pancreatic Cancer
PDAC:	Pancreatic Ductal Adenocarcinoma
Pen-Strep:	Penicillin-Streptomycin
PI:	Protease Inhibitor
PVDF:	Polyvinylidene Fluoride
qPCR:	Quantitative PCR
RIPA:	Radioimmunoprecipitation Assay Buffer

RNA:	Ribonucleic Acid
shRNA:	short hairpin RNA
TBST:	Tris-Buffered Saline with Tween 20

I. INTRODUCTION

1.1. Background Information

Pancreatic cancer (PC) is a highly lethal malignancy that originates in the exocrine or endocrine compartment of the pancreas. This cancer responsible for one of the most dismal prognosis among all cancer types (Ghafouri-Fard et al., 2021). The most common form of PC is, pancreatic ductal adenocarcinoma (PDAC), accounting for about 90% of all pancreatic cancer cases (Truong and Pauklin, 2021). PDAC is characterized by an aggressive phenotype and significant therapeutic resistance. This multidrug resistance (MDR), renders standard treatments ineffective which leads to an extremely poor patient prognosis, as evidenced, the five-year survival rate that is lower than 10% (Sarantis et al., 2021). Hence, MDR remains as one of the major challenge in treating pancreatic cancer (Masetto, 2022).

Although chemotherapeutic resistance can occur through a wide variety of different mechanisms, one notable source of resistance would be the adaptation of cancer cells into a polyploid giant cancer cell (PGCC) phenotype which are characterized by increased cell size and ploidy numbers. PGCCs exhibited a state of reversible cell cycle arrest which contribute significantly to the tumor's resilience against chemotherapy by promoting drug resistance, tumor recurrence, and metastasis (Jiao et al., 2022 & Mallin et al., 2023). While the mechanisms behind PGCC formation and their role in cancer progression are still unknown, preliminary study shows that the knockdown of Gene X resulted in the reduce of cells regeneration and forming a gigantic cells, which contrasts with this study that specifically investigates how Gene X knockdown influences the characteristics and drug resistance of PGCCs in pancreatic cancer. Interestingly, other studies in other cancers have pinpointed that Gene X possesses oncogenic functions, with its upregulation supporting uncontrolled cellular proliferation by inhibiting p21, a key cell cycle arrest protein. However, there is currently a lack of studies investigating the role of Gene X in causing PGCC formation in pancreatic cancer.

1.2. Research Aim

To elucidate the role of Gene X in the formation of PGCC and its impact on multidrug resistance in PDAC, which can be achieved by:

1. Determining the correlation between Gene X knockdown and giant cell formation as the main characteristic of PGCC.
2. Investigating the relationship between Gene X knockdown and multidrug resistance mechanisms in PDAC cells.

1.3. Scope of Research

To achieve the aims of this study, an in vitro investigation was conducted using pancreatic cancer cell lines with Gene X knockdown to explore its role in cellular responses. This approach involved establishing targeted Gene X knockdown models to evaluate its impact on multidrug resistance by assessing changes in cellular resistance to various chemotherapeutic agents. The study further quantified cell viability and proliferation rates in Gene X knockdown cells to determine its effects on survival and growth.

1.4. Hypothesis

Decreased expression of Gene X will promotes formation of PGCC, which exhibit enhanced resistance to chemotherapeutic agents in PDAC. This suggests that Gene X plays a pivotal role in modulating both the genomic stability of cancer cells and their susceptibility to chemotherapy.

II. LITERATURE REVIEW

2.1. Pancreatic Cancer (PC)

PC is recognized as one of the most lethal malignancies worldwide, with pancreatic ductal adenocarcinoma (PDAC) being the predominant subtype, accounting for approximately 90% of all pancreatic cancer cases (Mizrahi et al., 2020). The characteristics of PDAC include its aggressive nature, late-stage presentation, and a dismal five-year survival rate of less than 10% (Ghafouri-Fard et al., 2021). The high mortality rate associated with PDAC can be attributed to several factors, including its asymptomatic progression, which often leads to late diagnosis, and the tumor's intrinsic resistance to conventional therapies (Nevala-Plagemann et al., 2020).

Current treatment modalities for PDAC primarily include surgical resection, chemotherapy, and radiation therapy. Surgical resection offers the only potential for long-term survival; however, only about 20% of patients are eligible for surgery at the time of diagnosis (Mizrahi et al., 2020). Chemotherapy drugs, such as gemcitabine and nab-paclitaxel, are commonly employed but often yield limited improvements in overall survival due to the rapid development of chemoresistance (Ghafouri-Fard et al., 2021). This resistance is multifactorial, involving genetic, epigenetic, and microenvironmental factors that contribute to the tumor's ability to evade therapeutic agents (Zahan et al., 2020).

2.2. Mechanisms of Multidrug Resistance (MDR) in PDAC

MDR is a critical barrier to effective cancer therapy, defined as the ability of cancer cells to resist the cytotoxic effects of multiple chemotherapeutic agents (Patil et al., 2021). In PDAC, the emergence of MDR significantly complicates treatment regimens and is associated with poor patient outcomes. Furthermore, development of MDR also can promotes disease progression and metastasis by enabling cancer cells to survive and proliferate despite the presence of therapeutic agents, thereby

facilitating the spread of the disease to other tissues (Emran et al., 2022). Hence, understanding the keyplayer of MDR in PDAC is essential for developing targeted therapies that can effectively solve these resistance pathways and improve treatment responses.

The mechanisms underlying multidrug resistance (MDR) are diverse and can be categorized into intrinsic resistance, present before treatment, and acquired resistance, which develops during therapy (Ashrafizadeh et al., 2024). In pancreatic ductal adenocarcinoma (PDAC), several cellular mechanisms contribute to intrinsic resistance. For instance, cancer cells can upregulate efflux transporters, such as ATP-binding cassette (ABC) transporters, which actively pump chemotherapeutic agents out of the cell, thereby reducing drug accumulation and effectiveness (Zhou et al., 2020). Additionally, cancer cells may evade apoptosis, or programmed cell death, by upregulating anti-apoptotic proteins or downregulating pro-apoptotic factors, allowing them to survive despite cytotoxic treatment (Dandoti, 2021). Furthermore, alterations in drug targets can also be considered a form of intrinsic resistance; mutations in target genes can lead to structural changes in proteins that prevent effective drug binding, as seen with gemcitabine resistance in PDAC (Anand et al., 2023).

In contrast, acquired resistance mechanisms develop during therapy. For example, mutations in critical genes involved in drug metabolism can lead to changes in the pharmacokinetics of chemotherapy agents, affecting how the body absorbs, distributes, metabolizes, and excretes these drugs. Specifically, mutations in genes encoding drug-metabolizing enzymes, such as cytochrome P450 enzymes, can alter the rate at which a drug is broken down in the liver, resulting in increased toxicity due to drug accumulation or decreased efficacy if the drug is metabolized too quickly, preventing it from reaching therapeutic levels (Abdelmonem et al., 2024). Collectively, these mechanisms highlight the complexity of drug resistance in PDAC and underscore the need for further research into additional factors, such as the role of Gene X in giant cell formation, which may

contribute to both drug resistance and tumor progression. While understanding these mechanisms provides valuable insights into the challenges of treating PDAC, it is crucial to explore the role of Gene X in contributing to giant cell formation, as this may reveal additional pathways that influence both drug resistance and tumor progression.

2.3.Polyploid Giant Cancer Cells (PGCCs)

PGCCs are characterized by their giant cell morphology, which results from this chromosomal abnormality, this confers unique features that enhance their adaptability to environmental stresses (Pienta et al., 2021). The formation of PGCCs often arises from mechanisms such as mitotic slippage, endoreplication, and cytokinesis failure, which can be triggered by genotoxic stress or exposure to chemotherapeutic agents (Niu et al., 2017). These processes lead to the generation of cells with multiple sets of chromosomes, enabling them to exhibit increased genomic plasticity and resilience in the face of therapeutic challenges (Zhao et al., 2024). For instance, the failure of proper mitotic division can result in the accumulation of extra chromosomes, altering the genetic landscape of the cells and enhancing their ability to survive under stress (Erenpreisa et al., 2022). This genomic instability is a hallmark of many cancers, including pancreatic ductal adenocarcinoma (PDAC), and is associated with a more aggressive tumor phenotype.

The unique characteristics of PGCCs enable them to withstand chemotherapy-induced stress, leading to tumor resilience (Mallin et al., 2023). For example, the reversible cell cycle arrest exhibited by PGCCs allows for a survival advantage, enabling these cells to re-enter the cell cycle once the trigger is removed (Moein et al., 2020). This adaptability contributes to the high rates of relapse observed in PDAC patients following treatment. Moreover, PGCCs have been shown to possess stem-like properties, which further enhance their regeneration rate after therapy (Niu et al., 2017). The presence of PGCCs in the tumor microenvironment can create a group of cells that are capable of initiating new tumor growth, even after aggressive treatment. This phenomenon underscores the

importance of targeting PGCCs in therapeutic strategies aimed at improving long-term outcomes for PDAC patients.

2.4.Role of Gene X in Cancer Biology

Gene X has been implicated in cellular proliferation across various cancer types. Gene X contribution to tumorigenesis is mostly attributed to its known functions in regulating cell cycle progression and apoptosis, particularly through interactions with key proteins such as p21, which plays a crucial role in cell growth and survival (Dong et al., 2021). Additionally, Gene X may also influence tumor progression through other cellular processes, such as DNA repair and stress response. Understanding the multifaceted roles of Gene X in cancer biology may provide insights into its potential as a therapeutic target.

In the context of pancreatic ductal adenocarcinoma (PDAC), the hypothesis that Gene X plays a pivotal role in the formation of polyploid giant cancer cells (PGCCs) is supported by its involvement in cellular proliferation and cell cycle checkpoint regulation. This suggests that Gene X is not only essential for normal cell division but also for the aberrant growth patterns observed in cancer. Alterations in Gene X expression can lead to the development of PGCCs, which are characterized by their ability to evade apoptosis and exhibit enhanced survival under stress, contributing to multidrug resistance and promoting tumor progression in pancreatic cancer (Ji et al., 2022). Investigating the mechanisms by which Gene X influences these processes may provide valuable insights into potential therapeutic strategies for overcoming chemoresistance in PDAC..

III. MATERIALS & METHODS

3.1. Primers and shRNAs

The short hairpin RNA (shRNA) vector pLKO_TRC005 (Academia Sinica, Taiwan) was employed to achieve knockdown of Gene X. Two shRNAs were utilized: one specifically targeting Gene X and the other targeting Luc as a negative control for all subsequent analyses. Prior to transfection, the sequences of the shRNAs aimed at Gene X were confirmed using a U6 reverse sequencing primer. Additionally, a plasmid containing GFP was transfected to assess the transfection efficiency. The primers were designed using NCBI's primer BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), and the mRNA levels of Gene X were measured using qPCR, with normalization against GAPDH mRNA values.

3.2. Cell Culture and Maintenance

PANC-1 cells were maintained in DMEM with 10% Fetal Bovine Serum (FBS) (Merck, Taiwan) and 1% Penicillin-Streptomycin (Pen-Strep) (Cytiva, Taiwan) at 37^{o}C with 5% CO_2. The media was changed every 2-3 days to ensure optimal growth conditions and nutrient availability. Cells were passaged once they reached 80-90% confluency. For cell cryopreservation, 500µL of CELLBANKER® Cell Freezing Media (Amsbio, Taiwan) was used for each plate with 80-90% confluency.

3.3. Generation of Knockdown Cell Lines

The bacterial plasmids employed in transfection was amplified using LB agar and extracted in accordance to the kit directions of the Maxiprep plasmid extraction kit (Qiagen, Taiwan). When cells have reached ≥80% confluency in a 6-well plate, transfection was achieved using a 1:4 ratio between the shRNA containing plasmids and TransIT-X2 reagent. Specifically, co-transfection was achieved using a 7:3 ratio of shRNA containing plasmids and GFP-encoding plasmids which provide a quantitative measure of transfection efficiency. One shRNA controls targeting Luciferase was

employed in each batch of transfection. Additionally, a non-transfected control was utilised as a background control. Transfection was performed in one 6 well plate, which yielded a total of four Gene X clones of PANC1 (Supplementary figure 3) cells which have been cryopreserved using CELLBANKER® Cell Freezing Media (Amsbio).

In particular, a total mass of 2.5 µg of plasmid DNA and 15 µL of TransIT-X2 reagent were separately mixed into 300 µL of OptiMEM media, before the TransIT-X2 reagent was aliquoted into the tube containing plasmid DNA and allowed to incubate for 30 minutes to allow for the formation of plasmid TransIT-X2 complexes. Following incubation, the mixture was spun down before being added to the PANC-1 cells in droplets, and 2.7 mL of serum-containing media was added to the wells. The plates were then allowed to incubate for 48 hours before the media was substituted with fresh serum containing media. Following 48 hours of further incubation, puromycin selection was performed using complete media containing 6 µg/mL of puromycin. The cells were then allowed to be incubated for 48 hours before media was substituted with fresh media containing 3 µg/mL of puromycin, which was then routinely changed every 48 hours with fresh maintenance media containing 3 µg/mL of puromycin. Once KD cells reached sufficient confluence, they were transferred from the 6-well plate to a P60 plate, and subsequently, to a P100 plate, where they were expanded prior to cryopreservation.

3.4. Gene Expression Analysis

RNA extraction was performed using the TRIzol method. Samples were harvested in TRIzol reagent (Thermo Fisher, United States) and incubated at room temperature for 5 minutes to dissociate nucleoprotein complexes. Chloroform was added at a ratio of 1:5 to TRIzol, vortexed for 5 seconds, and incubated at room temperature for 2–3 minutes. The mixture was centrifuged at 12,000 × g for 15 minutes at 4°C, and the colorless RNA-containing supernatant was transferred to a new Eppendorf tube. Isopropanol was added at a ratio of 1:2 to TRIzol, mixed thoroughly, and incubated at -80°C

overnight to precipitate RNA. The samples were centrifuged at 12,000 × g for 30 minutes at 4°C, and the supernatant was discarded. The RNA pellet was washed twice with 1 mL of 75% ethanol (Honeywell, United States) prepared with DEPC-treated water, by centrifuging at 12,000 × g for 5 minutes at 4°C. After washing, the pellet was air-dried for 5–10 minutes until it turned transparent. The RNA pellet was dissolved in 30 µL of RNase-free water and incubated at 60°C for 10 minutes to ensure complete dissolution. The sample purity was then assessed before proceeding to cDNA synthesis.

Subsequently, cDNA was generated from 1 µg of total RNA using the iSCRIPT cDNA Synthesis Kit (Bio Rad, United States), following the manufacturer's instructions. Briefly, the protocol involved mixing the total RNA with the iSCRIPT reaction mix then incubated at 25°C for 5 minutes for primer annealing, followed by 42°C for 30 minutes for reverse transcription, and finally heated at 85°C for 5 minutes to inactivate the reverse transcriptase.

qPCR was performed using SYBR Green-based assays (Roche, Switzerland) targeting Gene X, with GAPDH serving as a housekeeping gene, on a Roche Real-Time PCR System. Each qPCR reaction had a total volume of 20 µL, consisting of 15 µL of SYBR Green (Roche, Switzerland) PCR Master Mix, 1 µL of forward primer, 1 µL of reverse primer, and 5 µL of the cDNA template. The thermal cycling protocol started with an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. The expression levels of Gene X mRNA were analyzed under two independent knockdown conditions, ShGene X #1 and ShGene X #2, and compared to the control group (Luc). The relative expression of Gene X was determined using the ΔΔCt method, and the results are presented as relative fold changes.

3.5. Protein Expression Analysis

Gene X protein expressions level was analyzed using Western blot technique. The process began with cell lysis with 1xRIPA buffer suplemented with 10 µL/mL protease inhibitors (PI) (400 µL for 10 cm plates and 200 µL for 6 cm plates), and the plates were incubated at 4°C for 30 minutes on a shaker. Cells were then scraped and transferred to new tubes. The samples underwent sonication (9–12 strokes or 3–6 seconds of continuous sonication, depending on viscosity) and were subsequently centrifuged at 14,000 rpm for 15 minutes. Protein concentrations were determined using the Bradford assay (Bio Rad, United States). A standard curve was prepared with protein concentrations ranging from 0 to 100 µg/mL. Samples were diluted 100-fold with injection water, and 900 µL of diluted Bradford reagent was added to each sample. Absorbance was measured at 595 nm.

Gel preparation included cleaning, drying, and assembling the gel-casting apparatus to prevent leakage. Running and stacking gels were prepared using acrylamide, TEMED, and APS, all kept on ice to ensure stability. The running gel was poured first and overlaid with ethanol or ddH2O to form a flat surface. After polymerization, the stacking gel was added, and a comb was inserted to eliminate bubbles. The gels were stored at 4°C in plastic wrap and ddH2O until further use.

Before electrophoresis, samples were heated to 95°C for 5 minutes. The gel was assembled in the Hoefer system, washed, and filled with running buffer. Samples were loaded into the wells, and the gel was run at 80V for 10–20 minutes until the running gel was reached. The voltage was then increased to 120V and continued for approximately one hour.

Lastly, for wet transfer, the gel was immersed in ddH2O, and the PVDF membrane was activated in methanol before equilibration in transfer buffer. A transfer sandwich was assembled in a container filled with transfer buffer, positioning the gel on the negative side and the membrane on the positive side. The transfer was carried out at 350 mA for one hour. The membrane was blocked in 1% milk for

one hour and washed three times with TBST (5 minutes each). It was then incubated at 4°C with the primary antibody targeting rabbit-anti-GAPDH and Gene X (GeneTex, Taiwan), respectively, which was diluted in 1:1000 ratio in 1 mL of blocking buffer for 48 hours. Afterward, the membrane was washed again with TBST and incubated with the donkey-anti-rabbit secondary antibody (Cytiva, Taiwan), diluted at 1:10,000 in 1% milk, for one hour. Following additional washes, the membrane was treated with ECL solutions (500 µL each of solutions 1 and 2) for 10 seconds. The resulting signals were captured and visualized. The relative protein expression of Gene X was measured under two knockdown conditions (ShGene X #1 and ShGene X #2).

3.6. Cell viability assay

The affect of Gene X knockdown on cell viability is done by employing MTT assay which began with seeding PANC-1 Gene X knockdown and PANC-1 Luciferase as control cells at 10000 cells per well in a 96-well plate, which was then incubated for 24 hours to allow cell attachment. The cells were then treated with varying concentrations of Gemcitabine (1, 4, 10, 20, 50, and 100 µM) and Paclitaxel (1, 4, 10, 20, 50, and 100 nM) for incubation periods of 24, 48, and 72 hours. After the drug treatments, the media were removed, and the wells were washed with 200 µL of 1x PBS. Subsequently, 200 µL of MTT reagent was added to each well, and the plate was incubated for four hours in the dark. The MTT reagent was then removed, and the wells were washed again with 200 µL of 1x PBS. Formazan crystals formed during the assay were solubilized by adding 100% DMSO. The absorbance was measured at a wavelength of 570 nm using a plate reader.

3.7. Data analysis

Data analysis was conducted using Microsoft Excel for statistical evaluation of the experimental results. For quantitative data, such as cell viability and gene expression levels, statistical significance was determined using appropriate statistical tests, including one-way ANOVA. Results were

expressed as mean ± standard deviation (SD) from at least three independent experiments, with a p-value of less than 0.05 considered statistically significant.

Western blot quantification and average cell size measurements were performed using ImageJ software. The intensity of protein bands was analyzed to determine relative expression levels of Gene X, while the average cell size was calculated based on pixel percentage measurements obtained from microscopic images. Statistical analysis was conducted using one-way ANOVA to assess differences between groups. Graphical representations of the data were created using Microsoft Excel to facilitate visual interpretation of the results.

IV. RESULTS AND DISCUSSION

4.1. Validation of Gene X knockdown through qPCR and Western Blot

To validate two different shRNA knockdown efficiancy on Gene x based on the mRNA expression of Gene X, qPCR was performed. The results shows a significant reduction in Gene X mRNA expression was observed in both ShGene X #1 and ShGene X #2 knockdown conditions relative to the Luc control, indicating successful knockdown of Gene X expression (Figure 1A). These findings are further validated by the decreases in Gene X protein levels by 52% and 63% upon the transfection of shGene X #1 and shGene X #2, respectively (Figure 1B), with ShGene X #2 ($p < 0.01$) demonstrated an even greater reduction than ShGene X #1 ($p < 0.05$)with high statistical significance. These results demonstrate that shRNA-mediated knockdown of Gene X effectively reduces its expression at both the mRNA and protein levels. The consistent reductions observed at both levels validate the specificity and efficiency of the shRNA constructs (Wang et al., 2023). Moreover, the greater reduction seen in ShGene X #2 suggests that this shRNA is more effective for targeting Gene X. This validation establishes a baseline for investigating the downstream effects of Gene X knockdown on cellular phenotypes, including morphological and functional changes.

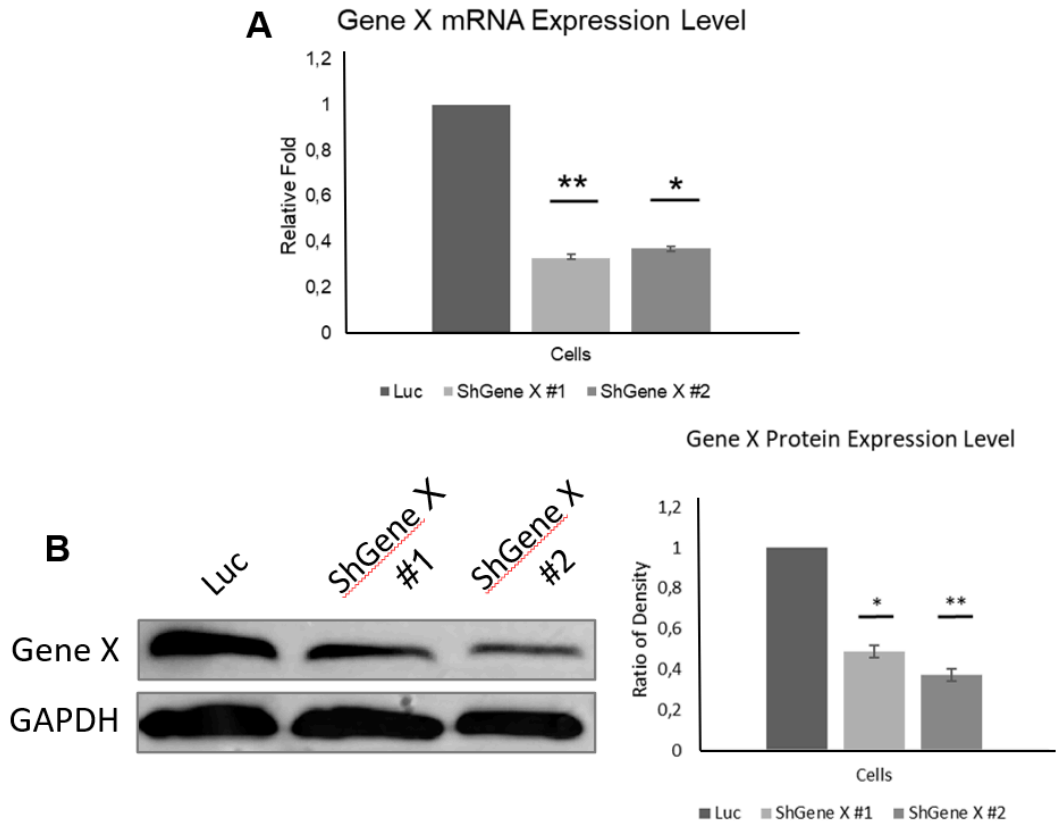

Figure 1. Validation of shRNA mediated Gene X knockdown at mRNA Expression Levels (A) and protein level (B) in knockdown cells compared to negative control cells. Results marked with a single asterisk (*) indicate statistical significance at $p < 0.05$, while double asterisks (**) denote $p < 0.01$.

4.2. Gene X Knockdown induces cellular giantism in PANC-1 cells

PGCCs are characterized by their giant cell size, polyploid state, and atypical structures, which typically emerge under conditions of cellular stress, genomic instability, or mitotic disruption (Casotti et al., 2023; Petersson, 2024). Given the role of Gene X in regulating cellular morphology and its potential implications in pancreatic ductal adenocarcinoma (PDAC), we investigated the effects of Gene X knockdown on PANC-1 cell size. In the Gene X knockdown conditions (ShGene X #1 and ShGene X #2), cells exhibited markedly enlarged sizes and irregular cellular morphologies, as indicated by white arrowheads. These phenotypic changes were absent in the control groups, including Luciferase-transfected (Luc) and non-transfected control (NTC) cells, which maintained

smaller and more uniform cellular sizes, typical of normal PANC-1 cells (Figure 2A). These observations is further supported by, quantitative analysis of average cell size was performed and expressed in pixels (Figure 3B). Cells from ShGene X #1 and ShGene X #2 knockdown groups displayed an increase in size, reaching ~300-400%. The enlarged cellular sizes and irregular morphologies observed in Gene X knockdown groups resemble features of Polyploid Cancer Cells (PGCCs). Together, these datas provide robust evidence that knockdown of Gene X induces cellular giantism and strongly suggests a role for Gene X in maintaining normal cellular size and structure.

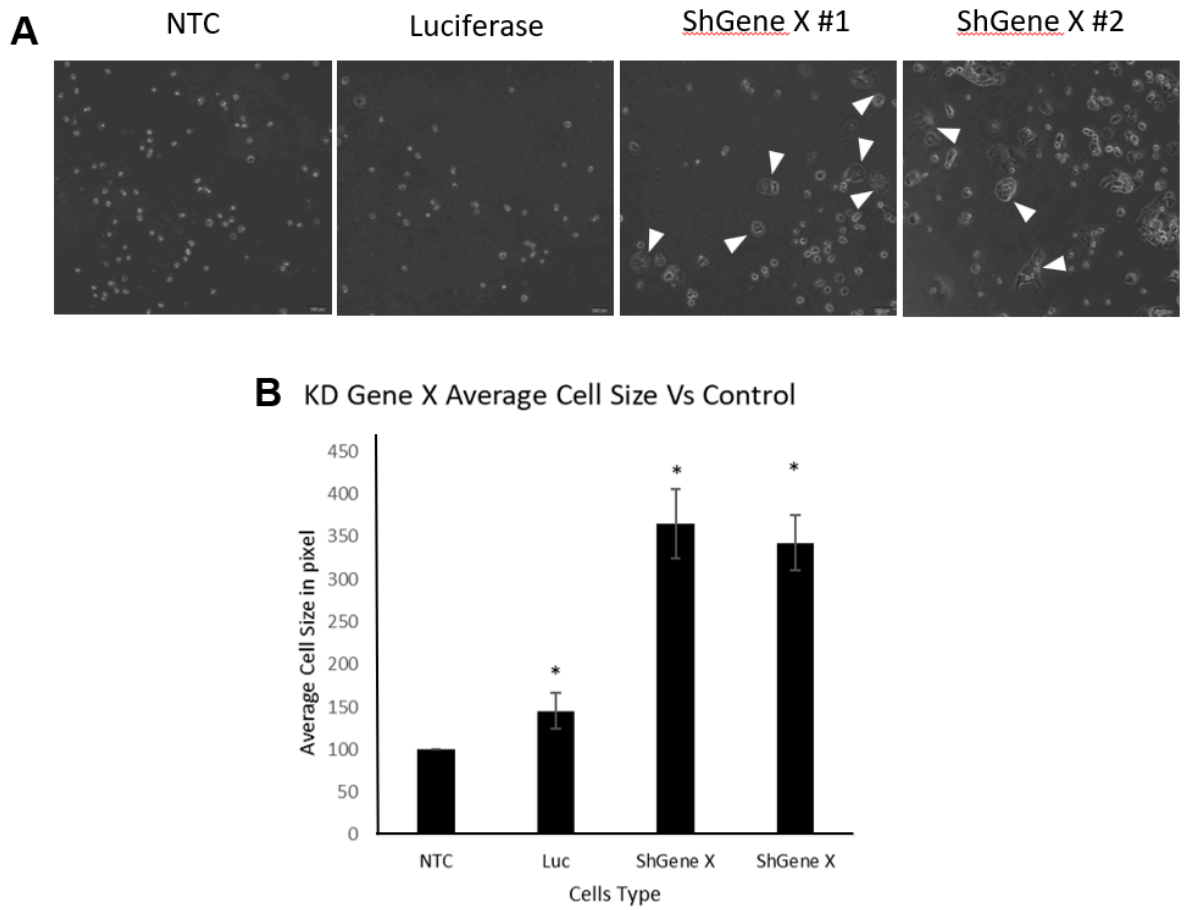

Figure 2. Figure 2. (A) Microscopic observations of the Gene X shRNA #1 and shRNA #2 knockdown (KD) clones, as well as the Luciferase and non-transfected controls, under 10x magnification. White arrows indicate giant cells. (B) Average cell size of Gene X knockdown in PANC-1 cells. Results marked with a single asterisk (*) indicate statistical significance at $p < 0.05$, while double asterisks (**) denote $p < 0.01$.

The phenotypic changes observed following Gene X knockdown can be attributed to multiple biological mechanisms. One possible cause of the observed cellular giantism is cell cycle dysregulation. Gene X may play a role in regulating key cell cycle processes, particularly during mitosis. Its depletion likely disrupts cytokinesis, leading to incomplete cell division (Kloc et al., 2022). In addition, loss of Gene X function may result in genomic instability, leading to chromosomal segregation errors during mitosis consistent with the observed phenotypes (Altamirano et al., 2021; Demers, 2020). However, to further validate the significance of Gene X knockdown in inducing PGCCs, additional studies, such as a metaphase spread assay should be conducted. This assay would allow for the examination of chromosomal abnormalities and provide direct evidence of the impact of Gene X depletion on chromosomal stability and the formation of polyploid cells.

4.3. The effect of Gene X knockdown towards multidrug resistance

To assess the effect of Gene X knockdown on PDAC viability, including the role of PGCC in resisting various chemotherapy agents, MTT assays were performed to evaluate the effects of Gemcitabine and Paclitaxel on cell survival in three groups: control (shLuc), shGene X #1, and shGene X #2. For Gemcitabine treatment (Figures 3A–C), the shGene X #1 and shGene X #2 groups showed slightly higher survival rates compared to the control group (shLuc) at all time points (24h, 48h, and 72h). However, the differences observed between the shGene X groups and the control group were not statistically significant, indicating that Gene X knockdown has only a minor impact on Gemcitabine sensitivity. In contrast, for Paclitaxel treatment (Figures 3D–F), the shGene X #1 and shGene X #2 groups demonstrated consistently higher survival rates than the control group, with this effect being more pronounced across all time points and drug concentrations. Notably, the resistant effect of Gene X knockdown is not directly observed at the 24-hour time point but becomes more evident at the 72-hour mark. This delayed response can be attributed to the time required for the cellular mechanisms involved in drug resistance to manifest fully (Marine et al.,2020). During the early phase of treatment, cells may still be undergoing mitotic arrest and initiating apoptotic pathways, which

can mask the effects of Gene X knockdown (Bai et al., 2023). As time progresses to 48 and 72 hours, the cumulative effects of prolonged exposure to Paclitaxel become more pronounced, allowing cells with knocked down Gene X to effectively bypass mitotic arrest and evade apoptosis, leading to a more significant increase in survival rates compared to the control group (Khing et al., 2021). This delayed manifestation underscores the importance of time in evaluating drug responses and highlights the complex interplay between Gene X knockdown and cellular survival mechanisms in the context of chemoresistance. These results suggest that the knockdown of Gene X contributes more significantly to Paclitaxel resistance compared to Gemcitabine.

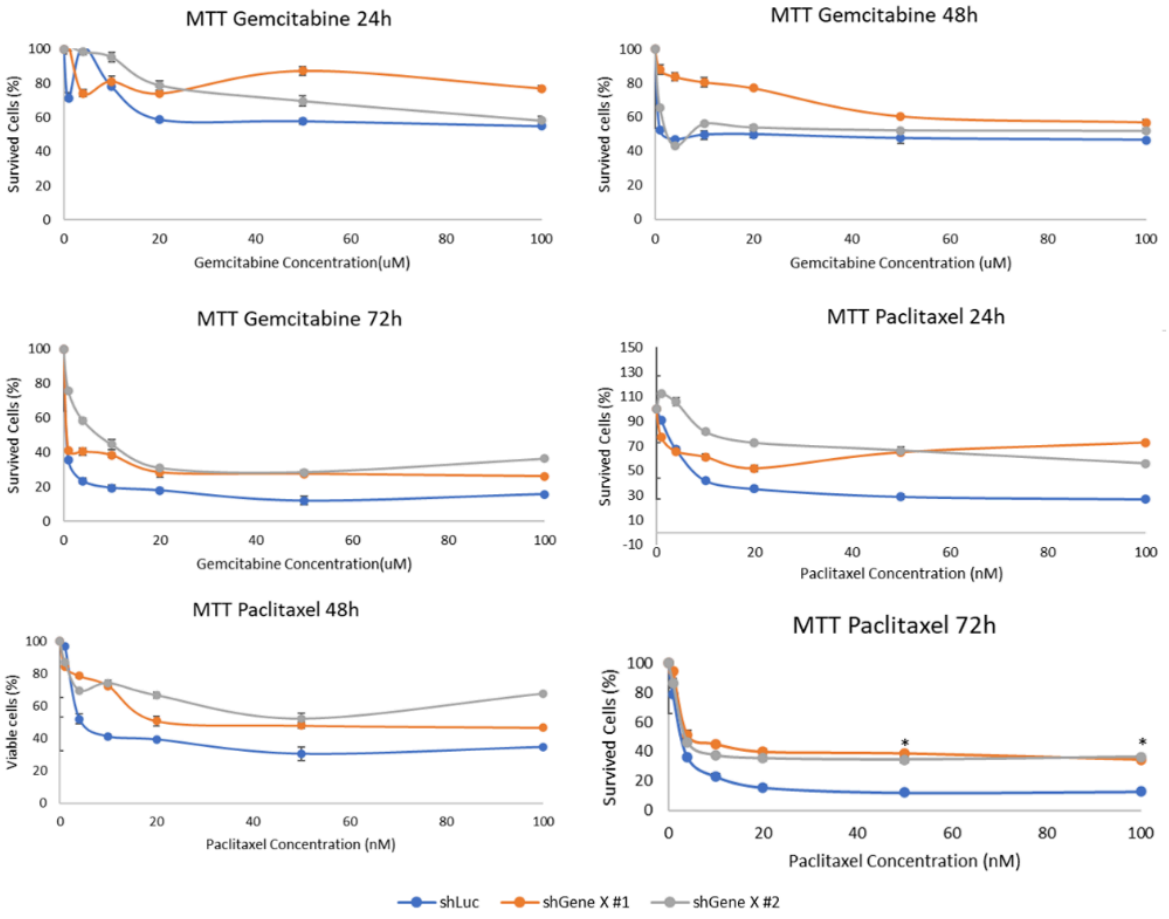

Figure 3. Effects of Gene X knockdown on cell survival following treatment with Gemcitabine and Paclitaxel. (A–C) Survival rates of cells treated with Gemcitabine at 24, 48, and 72 hours, (D–F) Survival rates of cells treated with Paclitaxel at 24, 48, and 72 hours.

This differential response to Paclitaxel and Gemcitabine highlights the likely role of Gene X in regulating cellular pathways specific to the action of each drug. Paclitaxel functions as a microtubule-stabilizing agent, preventing microtubule depolymerization and disrupting proper mitotic progression. As a result, cells exposed to Paclitaxel typically arrest in mitosis and initiate apoptosis (Sharifi-Rad et al., 2021). If Gene X is involved in regulating mitotic spindle assembly, checkpoint control, or apoptotic signaling, its knockdown could allow cells to bypass mitotic arrest or evade apoptosis, thereby increasing their survival under Paclitaxel treatment. This would explain why resistance to Paclitaxel is more prominent in cells where Gene X has been knocked down.

In comparison, Gemcitabine acts as a nucleoside analog that inhibits DNA synthesis during the S-phase of the cell cycle, leading to stalled replication forks and DNA damage (Gu et al., 2023). Since Gene X plays a role in DNA replication, its knockdown may interfere with the proper progression of replication forks or the cellular response to replication stress. This disruption could potentially worsen the replication stress caused by Gemcitabine (Segeren & Westendorp, 2022). However, the observed lack of significant differences in survival between the shGene X groups and the control group suggests that cells may activate compensatory mechanisms to counteract the loss of Gene X. For instance, upregulation of alternative replication stress response or DNA repair pathways could mitigate the impact of Gene X knockdown, allowing cells to survive Gemcitabine treatment (Zhang et al., 2022). This ability to compensate for Gene X loss may explain why the survival differences between the shGene X groups and the control group remain small and statistically insignificant when treated with Gemcitabine.

4.4. Limitation

This research offers valuable insights into the role of Gene X in relation to PGCCs and multidrug resistance (MDR). However, it is important to acknowledge several limitations that could be addressed in future studies. One limitation is the reliance on in vitro models, which may not fully

replicate the complex tumor microenvironment found in vivo, potentially affecting the generalizability of the results. Additionally, the study primarily focused on two specific shRNA constructs for Gene X knockdown, which may not encompass the full spectrum of Gene X functions or its interactions with other cellular pathways. Furthermore, the relatively short incubation periods for drug treatments may not capture the long-term effects of Gemcitabine and Paclitaxel on cell survival and resistance mechanisms. Another limitation is that this study only encompasses phenotypic observations of cell morphology, which cannot determine the polyploid factor associated with PGCCs. To further validate the findings of this study, flow cytometry could be employed to enhance our understanding of how Gene X induces PGCC formation. Extending the incubation periods may also provide deeper insights into the long-term effects of these chemotherapeutic agents.

V. CONCLUSION

This research establishes that Gene X plays a pivotal role in regulating both cellular morphology and drug resistance in Pancreatic Ductal Adenocarcinoma (PDAC) cell lines. The effective knockdown of Gene X resulted in significant reductions in its expression at both mRNA and protein levels, leading to pronounced cellular giantism and irregular morphologies. Additionally, the study highlights the differential impact of Gene X knockdown on drug sensitivity, with a notable increase in resistance to Paclitaxel, suggesting its involvement in critical cellular pathways related to mitotic progression and apoptosis. Future studies should aim to further elucidate the mechanisms by which Gene X influences polyaneuploidy cancer cell characteristics and explore the potential for targeting Gene X in therapeutic strategies to overcome multidrug resistance. Employing techniques such as flow cytometry and extending drug exposure periods could provide deeper insights into the long-term effects of Gene X knockdown on cellular behavior and treatment responses.

SELF REFLECTION

Throughout this experience working in Taipei Medical University under the guidance from my EP advisor Dr. Chen Hsin-Yi, B.Sc., M.Sc., Ph.D., I have acquired and improved a wide range of laboratory techniques that will be crucial for my future career in biomedical research. I developed strong analytical skills through data analysis and interpretation, which are crucial in any research-oriented role. I learned to utilize software tools for statistical analysis, enhancing my proficiency in data management and visualization. Additionally, I have also significantly improved my Western blot technique, which will be invaluable in molecular biology and clinical research Moreover, I honed my communication skills, both written and verbal, by preparing reports and presenting findings to my team. This experience taught me how to convey complex information clearly and effectively, a skill that is essential in any professional environment.

During my internship, I identified several strengths and weaknesses. One of my key strengths was my adaptability; I was able to adjust to new tasks and environments quickly, which allowed me to contribute effectively to various projects. My enthusiasm for learning also helped me to engage with my colleagues and seek feedback, fostering a collaborative atmosphere. However, I recognized that my weakness is sometimes not paying enough attention to details, which can lead to failed experiments. I learned the importance of being meticulous in my work and will strive to improve this aspect in my future endeavors.

The values of i3L—Grit, Role-Model, and Integrity—played a significant role in shaping my internship experience. Grit encouraged me to persevere through challenges and remain committed to my goals, even when faced with obstacles. This resilience was crucial in maintaining my motivation and focus throughout the internship. Being a Role-Model inspired me to lead by example, fostering a positive work environment and encouraging my peers. I strived to embody integrity in all my interactions,

ensuring that I approached my work ethically and responsibly. These values not only enhanced my personal growth but also contributed to a supportive and productive workplace culture.

The molecular oncology courses I took during my fifth semester significantly contributed to my success during the internship, as they provided me with the necessary background knowledge to understand the concepts behind my project and analyze the results obtained based on that understanding. Additionally, the laboratory courses from my earlier semesters introduced me to the fundamental laboratory rules and techniques required, allowing me to adapt well despite being in an advanced research lab. Furthermore, the BRIGHT Sessions offered by i3L were aldo invaluable for my soft skills development. These sessions focused on enhancing interpersonal skills, emotional intelligence, and leadership qualities. The interactive nature of these workshops allowed me to practice and refine my skills in a supportive environment, which translated into greater confidence and effectiveness in the workplace.

I believe my presence in the workplace had a positive impact on my team. My eagerness to learn and contribute fostered a collaborative spirit, and I was able to bring fresh perspectives to ongoing projects. By actively participating in discussions and offering insights, I contributed to a culture of innovation and continuous improvement.

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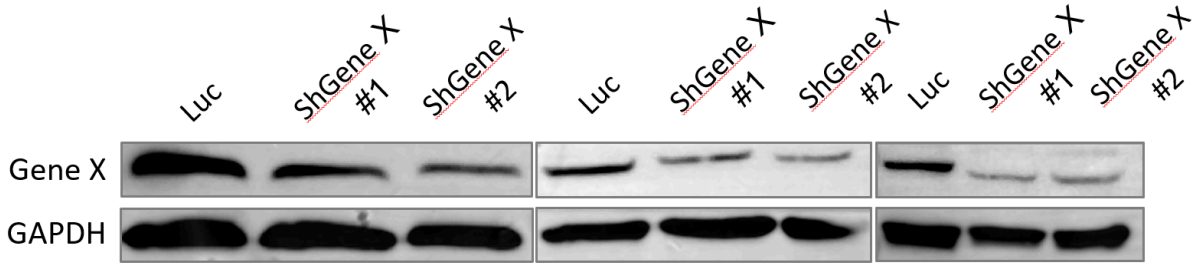
APPENDICES

Supplementary table 1. FASTA sequence of Gene X shRNA and primers, as well as GAPDH primers

Primers/ shRNA	FASTA Sequences
Gene X shRNA #1	CCGGTCGGCAATAGCTCACCGTTTACTCGAGTAAACGGTGAGCTATTGCCGATTTTTG
Gene X shRNA #2	CCGGAGTGCCGCTGAAGATCGCATTCTCGAGAATGCGATCTTCAGCGGCACTTTTTTG
Gene X Primer (F)	CGTGTGAAGGACCTGGAGTC
Gene X Primer (R)	CTGCTTTTTGGCCACTGCAT
GAPDH Primer (F)	GTCTCCTCTGACTTCAACAGCG
GAPDH Primer (R)	ACCACCTGTTGCTGTAGCCAA

Supplementary table 2. Complete western blot image used for band intensity quantification



Supplementary table 3. Transfection Mapping