Development of Gemcitabine Resistance in Pancreatic Adenocarcinoma

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1 | Introduction

1.1 | Chemoresistance in Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of tumour-related mortalities[1]. Despite decades of research, the 5-year overall survival is still less than 5%, which can be attributed to late detection, resistance to chemotherapy, aggressive tumour biology, and lack of personalized treatment[1,2]. Gemcitabine (GEM) is the first-line chemotherapeutic agent for PDAC and has improved the prognosis of patients over the past decade[1,3]. This cytidine analogue (20,20-difluoro-20-deoxycytidine) is incorporated into genomic DNA and acts by arresting DNA replication and cell growth[1,4]. The resistance to GEM is an intractable clinical problem and develops within weeks of initiation of therapy[1,4]. Therefore, a thorough understanding of the mechanisms underlying GEM chemoresistance is needed to improve its therapeutic efficacy[2,4].

Several molecular mechanisms have been elucidated, with increasing evidence indicating that microRNAs (miRNAs) regulate this drug resistance[3,5]. miRNAs are endogenous, single-stranded, non-coding RNAs, composed of 18–25 nucleotides[3]. As post-transcriptional regulators, miRNAs downregulate gene expression by binding directly to target mRNAs, thereby inducing their degradation or repression of translation[5]. Currently, there are several miRNAs involved in tumour resistance to GEM-based chemotherapy; Iwagami et al. performed a comprehensive expression profiling of miRNAs and identified miR-320c as one of the most commonly upregulated in GEM-resistant (GR) MiaPaCa2 and PSN1 cells (PDAC cell lines). The results also indicated that miR-320c conferred resistance through the SMARCC1 protein[3,5].

1.2 | The Role of Exosomal miRNAs in Gemcitabine Resistance

Tumours and their microenvironments contain multiple types of cells, such as adult stem cells, cancer stem cells (CSCs) and stromal cells[4]. The tumour microenvironment plays a vital role in the development of drug resistance and exosomes (Exo) may mediate the interactions among the different cell types[1,4]. These lipid bilayer vesicles with 30 - 100 nm in diameter are secreted to the extracellular space by various cell types, including tumour cells, and are taken up by neighbouring or distant cells[4,5]. Their main function is to participate in cell-cell communication by transferring biologically active molecules, such as proteins, DNA, mRNAs, and miRNAs to recipient cells[2,4].

Recent studies have shown that exosomes regulate GEM-chemoresistance in PDAC, by transferring miRNAs from M2 macrophages[1], CSCs[4] and cancer-associated fibroblasts (CAFs)[2] to recipient tumour cells. Binenbaum et al. recently showed that macrophage-derived exosomes significantly decreased the GEM sensitivity of the K989 cell line and that this effect was mediated by miR-365[1]. Additionally, Yang et al. demonstrated that exosomes derived from GR CSCs transferred drug-resistant traits to GEM-sensitive (GS) BxPC-3 and PANC-1 cells by delivering miR-210[4]. Furthermore, Fang et al. verified that

CAFs induced GEM resistance of neighbouring AsPC-1 and PANC-1 cells by transferring exosomal miR-106b[2].

Given that chemoresistance cannot be induced in all tumour cells simultaneously, recent evidence also indicates that exosomal miRNAs derived from drug-resistant cancer cells can transmit chemoresistance to drug-sensitive cancer cells[4,5]. Wei et al. demonstrated for non-small cell lung cancer (A549 cell line) that miR-22-3p was upregulated in both GR-cancer cells and their derived exosomes. Upon exosome internalization, miR-222-3p enhanced the proliferation, GEM resistance, migration, and invasion of GS-cancer cells by activating the SOCS3/Stat3 signalling pathway[5].

To date, this mechanism has not been reported for PDAC. In this experimental design, we propose that exosomes released by GR-cancer cells decrease the drug sensitivity of neighbouring and distal GS-cancer cells by delivering miR-320c, which is upregulated as previously described[3]. Because the exosomal miRNA profile does not resemble that of donor or recipient cells completely, additional candidate miRNAs differentially expressed between GR- and GS-derived exosomes will be identified through microarray analysis[5].

2 | Experimental Design

2.1 | Establishment and characterization of GR cell clones

In these experiments, the same human PDAC cell lines as Iwagami et al. will be used: MiaPaCa2 and PSN1[3]. The cells will be cultured according to the protocol provided by Iwagami et al.[3]. Firstly, GR-clones of the parental (P) cell lines will be generated. Therefore, the GS MiaPaCa2 and PSN1 cells will be gradually exposed to increasing concentrations of the drug for 2 months[3]. This process will be repeated three times for each P-cell line, obtaining the clones denominated as GR-1, GR-2, and GR-3.

To confirm the drug resistance of the obtained cell clones, the GR- and P-cancer cells will be cultured with increasing concentrations of GEM or the same amount of culture medium as the control. The cell viability for each concentration will be assessed using the MTT colourimetric assay to build a growth curve. For each cell line and GEM concentration, triplicate assays will be performed.

Lastly, the P- and GR-cell lysates will be analysed by Western blot for the drug resistance-related proteins MDR1, YB-1 and BCRP after exposure to different concentrations of GEM[4]. The actin protein will be used as a control and this assay will be done in duplicate.

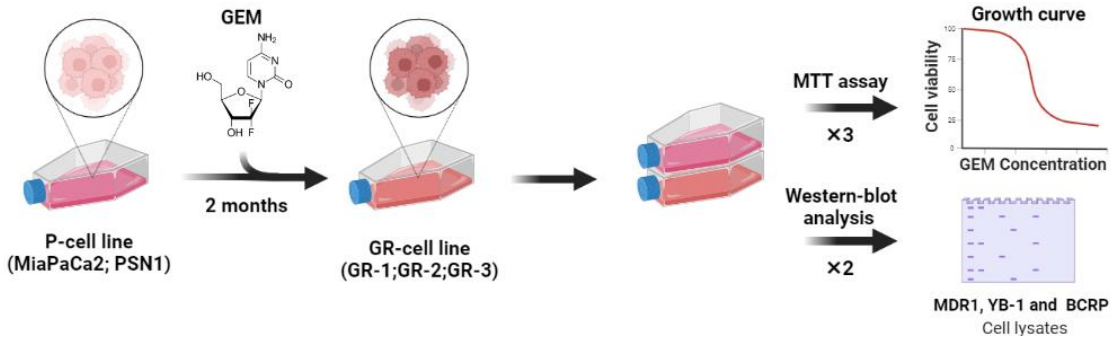

Figure 1: Generation and characterization of GR-cell clones. GR clones will be obtained from the parental cell lines (MiaPaCa2 and PSN1) by exposure to increasing concentrations of GEM and their chemoresistance characterized by Western blot analysis and MTT assays.

2.2 | Assessment of the effect of GR-derived exosomes on chemoresistance

Exosome isolation will be performed in triplicate as described by Yang et al.[4], from the supernatants of each P- and GR-cell line. The exosomal proteins will be extracted with a radioimmunoprecipitation assay lysis buffer, and a Western blot (in duplicate) will analyse the expression of exosome markers CD63, CD81 and GM130[4]. The presence of these markers will also be assessed in the cell lysates from which the vesicles were derived, to confirm exosome isolation efficiency. Exosomal morphology will also be assessed through transmission electron microscopy (TEM)[4].

Next, the effects of GR-derived exosome uptake on the proliferation and GEM resistance of P-cells will be evaluated. For that, these cells will be treated with different concentrations of GEM (or control) for 72 h, in the absence or presence of either P-Exo or GR-Exo. It is necessary to confirm if P-cells can uptake P- and GR-derived exosomes, which will be done by fluorescence labelling of the vesicles (as described by Yang et al.[4]). Their growth will be assessed by MTT assays as previously described[4]. A triplicate assay will be performed for each exosome and cell line combination (of the same parental cell origin), as well as for each concentration of GEM. Finally, a Western blot analysis of the cell lysate (in duplicate) will evaluate the expression of the drug resistance-related proteins (MDR1, YB-1 and BCRP) in the P-cells[4].

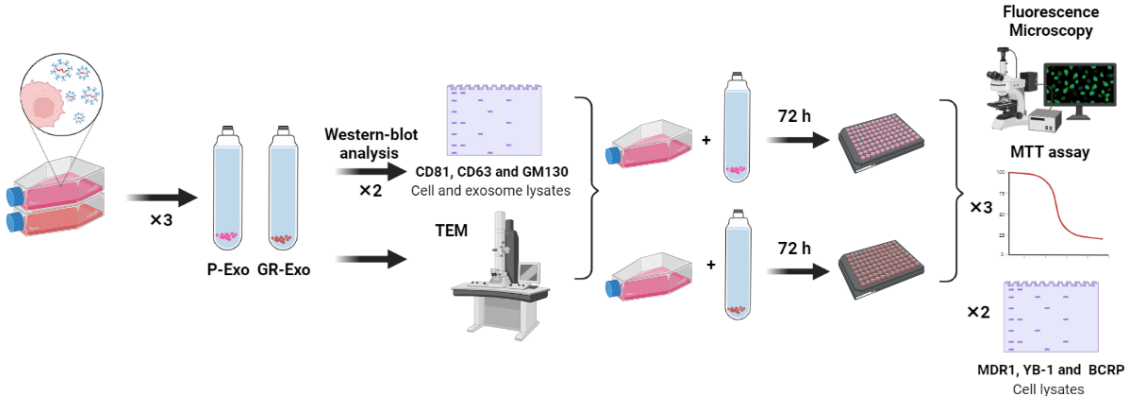

Figure 2: Effect of GR-derived exosomes on GEM chemoresistance. P- and GR-Exo will be isolated, their protein expression assessed by Western blot and their morphology analysed by TEM. After that, the isolated exosomes will be incubated with GS P-cells. Exosome uptake will be visually confirmed by fluorescence, GEM resistance assessed through MTT assays and resistance protein expression analysed by Western blot.

2.3 | Identification and functional validation of exosomal miRNA

After confirming the positive effect of GR-derived exosomes on chemoresistance, total RNA enriched with miRNAs will be isolated from cells and exosomes, as described in Yang et al[4]. The upregulation of miRNA-320c in both GR-cancer cells and their derived exosomes will be examined in duplicate by qRT-PCR to confirm our hypothesis. Simultaneously, a microarray analysis will be conducted using combined P- and GR-derived exosomes, to identify the miRNAs showing the highest alteration and statistical significance. The most upregulated miRNA will be selected for further experiments.

The candidate exosomal miRNA involved in tumour resistance will be functionally validated by transfecting (in duplicate) the corresponding pre-miRNA or a scrambled oligonucleotide (negative control) into the P-cell lines. Duplicate assays of qRT-PCR will be performed to confirm the successful transfections after 24h, 48h and 72h. Lastly,

triplicate MTT assays of the P-cell lines exposed to different concentrations of GEM in the absence or presence of the pre-miRNA (or negative control) will confirm and provide a possible mechanistic explanation for the observed positive effect of the GR-derived exosomes. Furthermore, a Western blot analysis of the cell lysates (in duplicate) will evaluate the expression of the drug resistance-related proteins in the transfected P-cells[4].

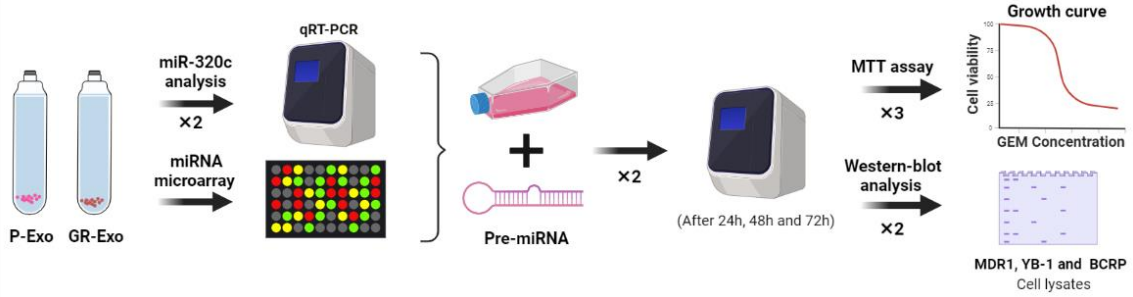

Figure 3: Identification and functional validation of exosomal miRNA. Firstly, the upregulation of miR-320c in GR-Exo will be analysed by qRT-PCR and additional candidate miRNAs identified through microarray analysis. The candidate exosomal miRNA will be functionally validated by transfecting the corresponding pre-miRNA into P-cells and the transfection confirmed by qRT-PCR. GEM resistance after transfection will be assessed through MTT assays and Western blot analysis of drug resistance proteins.

3 | Expected results and concluding remarks

Drug resistance is the major cause of treatment failure in cancer, yet the multifactorial mechanisms responsible for resistance remain largely unknown[3]. We expect that this experimental design demonstrates that exosomes derived from GR MiaPaCa2 and PSN1 cells decrease the drug sensitivity of the GS P-cells. As observed for non-small cell lung cancer, this effect in PDAC would be mediated by the transfer of a specific miRNA that promotes the malignancy[5]. Future studies are required to predict the putative targets of the miRNA (using bioinformatics tools such as TargetScan) and to evaluate the miRNA's potential as a prognostic biomarker for patients' response to therapy[3]. Furthermore, the transfection of antagomirs could be explored as a new therapeutic approach[1].

In spite of the novelty of our study, some limitations should be addressed. The protocol optimization process can be time-consuming, and our findings *in vitro* may not be translatable into *in vivo* conditions. Additionally, due to the intrinsic heterogeneity of cancer, different GEM resistance mechanisms can occur, and further experiments should be performed in other PDAC cell lines[6]. Alternatively to the MTT assay, other more expensive cell viability assays such as the MTS, which was not used in previous studies, could result in more accurate results[7].

Despite these possible challenges, evidence in PDAC and other cancers supports the feasibility of our project proposal. The obtained results will contribute to the understanding of GEM-chemoresistance, which severely limits the therapy's effectiveness, and contribute to ongoing research focused on preventing PDAC diagnosis from being a death sentence.

4 | References

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