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Preface

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Chapter 1

Abstract

Cancer cells exhibit a characteristic ability to evade programmed cell death, a feature critical for their uncontrolled proliferation. An emerging strategy in cancer therapy involves targeting alternative forms of cell death, distinct from apoptosis, as evidenced by recent studies (Tait, Ichim, and Green, 2014). Non-apoptotic cell death mechanisms, including necroptosis and pyroptosis, offer promising avenues to circumvent apoptosis resistance in tumors, thus enhancing the efficacy of anticancer therapies (Ye et al., 2018).

One such non-apoptotic pathway, ferroptosis, reliant on iron and reactive oxygen species (ROS), represents a novel approach in cancer treatment. However, inherent resistance to ferroptosis induction poses a challenge in its therapeutic application (Zou and Schreiber, 2020). Detecting drug resistance before treatment initiation could significantly impact treatment outcomes (Volm and Efferth, 2015).

Epigenetic modifications, such as DNA methylation, RNA methylation, and noncoding RNAs, have emerged as key regulators of cellular processes. Notably, DNA methylation alterations in genes associated with ferroptosis, including GPX4, FSP1, and NRF2, influence their expression levels, potentially modulating ferroptosis susceptibility (X. Wang et al., 2023).

Bisulfite genomic sequencing is widely utilized for DNA methylation detection, despite its limitations. Challenges such as incomplete bisulfite conversion or assay failures may compromise data accuracy (Li and Tollefsbol, 2011; Heiss and Just,

2018).

Recent advancements in sequencing technologies, such as nanopore sequencing, offer improved accuracy and resolution compared to traditional methods. Nanopore episequencing, in particular, shows promise in refining genetic associations and identifying novel loci previously undetected by microarray platforms (Gombert et al., 2023; Flynn et al., 2022).

In this project, we will aim to develop a machine learning model which, based on a nanopore episequencing profile, will be able to predict the likelihood of ferroptosis resistance for a given tumor strain. The model's capacity to generalize to other (untrained) strains will be tested. Lastly, we will identify specific methylation changes brought about by ferroptosis-inducing drugs by sequencing sensitized strains.

Chapter 2

Introduction

2.1 The occurrence of ferroptosis

Ferroptosis is a complex process with many contributing, interconnected factors. At its core, it is an intracellular iron-dependent form of cell death that is distinct from apoptosis, necrosis, and autophagy (Yan et al., 2021). As the name suggests, this phenomenon is principally driven by iron molecules absorbed by cells from the surrounding extracellular environment. One such receptor is transferrin receptor 1 (TFR1), which mediates endocytosis of diferric transferrin (S. Wang et al., 2019). This intracellular iron is often transported and stored in storage proteins such as ferritin, aiding in maintaining iron homeostasis and metabolism (Knovich et al., 2009).

Labile iron, however, which refers to loosely bound or chelatable iron, becomes a critical player in the ferroptotic process. Labile iron participates in the Fenton reaction, leading to the generation of reactive oxygen species (ROS), particularly hydroxyl radicals (Cabantchik, 2014). ROS, in turn, contributes to oxidative stress within the cell.

Polyunsaturated fatty acids in cellular membranes are highly susceptible to oxidative damage by ROS (Ayala, Muñoz, and Argüelles, 2014). Lipid peroxides are formed as a result of the oxidation of these fatty acids. Accumulation of lipid peroxides disrupts the integrity of cellular membranes, particularly the lipid bilayer

(Gaschler and Stockwell, 2017). Membrane damage leads to the loss of compartmentalization and function within the cell, including the mitochondrion (Ademowo et al., 2017).

Healthy mammalian cells stave off this threat in a number of ways. The primary cellular mechanism of protection against oxytosis/ferroptosis is mediated by glutathione peroxidase 4 (GPX4), a glutathione-dependent hydroperoxidase that converts lipid peroxides into non-toxic lipid alcohols (Xie et al., 2023). Glutathione itself, which is produced from cysteine, is able to neutralize ROS present within the cell directly, playing the role of antioxidant (Averill-Bates, 2023).

Even when glutathione peroxidase functionality is uncompromised, research has shown that the mitochondrion plays a substantial role in ferroptosis regulation and induction, being the major compartment for cellular iron metabolism (Liu et al., 2023). Mitochondrial shrinkage and rupture are also common observations when a cell undergoes ferroptosis, since this organel is enveloped in a membrane (Gao et al., 2019).

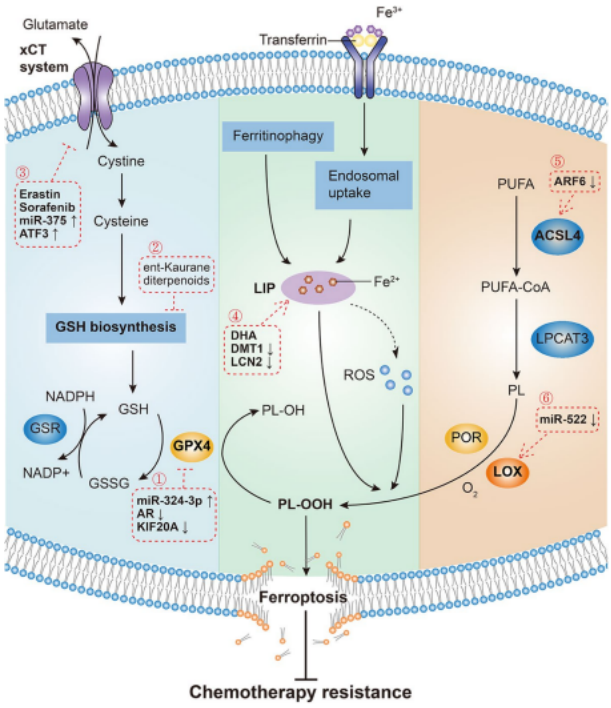

Figure 2.1: Figure depicting the various regulatory pathways associated with ferroptosis (Zhang et al., 2022a).

2.2 Ferroptosis in cancer therapy

Cancer is the second leading cause of death worldwide, behind cardiovascular disease (H. Wang et al., 2016). However, improvements in cancer survival rates due to the use of precision medicine or immunotherapy

There are a number of ways to counteract this resistance, including inhibition of glutathione synthesis, overexpression of transferrin receptors, underexpression of or defective ferritin (including the chaperone proteins that fill the ferritin) and/or ferroportin proteins (which export labile iron bound to chaperones outside the cell) (Zhang et al., 2022b).

2.3 Oxford Nanopore Episequencing

Nanopore sequencing is a next-generation sequencing technology that enables the real-time analysis of DNA or RNA molecules as they pass through a nanoscale pore. This technique is based on the principle that as a DNA strand translocates through a nanopore, changes in the electrical current can be measured and used to deduce the sequence of the DNA molecule Y. Wang et al., 2021. The raw electrical signal is translated into DNA sequence information through a process called basecalling. DNA methylation involves the addition of a methyl group to a cytosine base (5-methylcytosine). Nanopore sequencing can detect these modifications as changes in the electrical current during translocation using specialized algorithms and basecallers. One of the key advantages of nanopore sequencing is its ability to generate long reads, providing information about entire genomic regions in a single continuous sequence.

Adaptive sampling is a feature associated with PromethION that helps optimize the sequencing process by adjusting data collection parameters in real-time based on the characteristics of the DNA being sequenced. The system monitors various parameters such as the quality of the signal, the speed at which the DNA is translocating through the nanopore, and other relevant metrics (Martin et al., 2022). The software requires a user to upload a file of whitelisted reference sequences, and

the system can be set to either deplete or enrich for these on a specified set of channels.

In order to achieve this, the software basecalls the first few hundred bases of each read and compares it with the target reference sequences. Matching or unmatching sequences are rejected, depending on whether the software is set to enrich or deplete. By using this, coverage of our genes of interest is enriched, greatly reducing noise.

Chapter 3

Aims

Chapter 4

Methods

4.1 Introduction

Firstly, differential gene expression analysis was performed. Then, feature reduction of the base-level modification information obtained from ONT episequencing was carride out. Finally, we trained a predictive model using the reduced feature matrix.

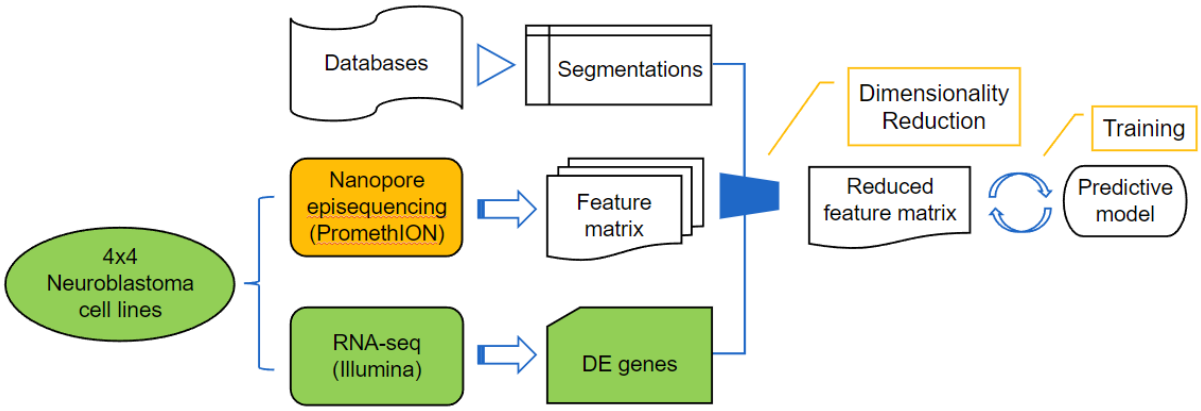

Figure 4.1: Overview workflow.

4.2 Samples

To start off, expression data from 4 cell lines was gathered, namely 2 resistant and 2 sensitive neuroblastoma cell lines in quadruplicate (16 samples in total). These cell lines were characterized in terms of sensitivity towards ferroptosis beforehand experimentally. Furthermore, neuroblastoma is highly iron addicted which makes it exceptionally vulnerable to ferroptosis (Floros et al., 2021). Metadata for each cell line can be found in Table 4.1.

Cell Line	Tumor Type	Ferroptosis Sensitivity	Gender	Age (months)
IMR-32	Neuroblastoma	Sensitive	Male	13
SH-EP	Neuroblastoma	Sensitive	Female	48
SH-SY5Y	Neuroblastoma	Resistant	Female	48
SK-N-BE(2)-C	Neuroblastoma	Resistant	Male	26

Table 4.1: Cell Line Metadata

4.3 Gene Whitelist

Genes thought to be associated with ferroptosis were compiled into a custom gene whitelist. The full list can be found in Appendix X.

4.4 RNA-seq

4.5 ONT-seq

For the episequencing, a PromethION nanopore sequencing device based at OHMX.bio was used, containing 24 channels per flow cell. PromethION offers a significantly higher number of channels compared to other ONT platforms like MinION and GridION. This high channel count enhances the platform's capacity for parallel sequencing.

From the whitelist, a BED file was constructed. Additionally, each gene was extended by 15kB, after which overlapping genes were joined together.

Library prep involved following protocol SQK-NBD114.24, describing how to carry out native barcoding of genomic DNA (gDNA) using the Native Barcoding Kit 24 V14. Raw signal data in pod5 format was basecalled with a SUP model and 5(h)mC calling in CG context using GPU hardware on a GridION unit installed at OHMX.bio, and then subsequently demultiplexed. The basecalling model was set to fast, with the intent of making the decision of whether to reverse the voltage of the pore as fast as possible. The resulting unmapped bams were then aligned against a reference genome, and finally sorted and indexed using SAMtools (Danecek et al., 2021).

4.6 Differential Gene Expression Analysis

4.7 Feature Reduction

Since the episequencing returns many thousands of individual detected CpGs by locus, the number of features in our model will be staggering if used in its raw form. The first reduction of features will be actualized by bundling CpG sites into regions (e.g. CpG islands). A second dimensionality reduction will also be performed based on drivers derived from expression data, to remove sites or regions without genes that are differentially expressed by sensitivity.

Chapter 5

Results

Chapter 6

Discussion

Appendix A

Appendix

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