Targeting MRP2 in HepG2 Cells Using the CRISPR-Cas9 System to Reverse Oxaliplatin Resistance

Ying Zou

A thesis submitted to the Faculty of Health and Environment Sciences in partial fulfilment of the requirements for the degree of Master of Science, Auckland University of Technology, 2019

Targeting MRP2 in HepG2 Cells Using the CRISPR-Cas9 System to Reverse Oxaliplatin Resistance

Approved by:

Supervisors: Dr. Yan Li (Primary Supervisor)

Dr. Dong Xu Liu (Secondaty Supervisor)

Table of Content

Contents

List of Figures

List of Tables

Attestation of Authorship

I hereby declare that this submission is my 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), or material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or institution of higher learning.

Signed

Name: Ying Zou

Date: 5th July, 2019

Abstract

As a third-generation platinum-based anticancer drug, oxaliplatin is critically important in the treatment of hepatocellular carcinoma (HCC), colorectal (CRC) and other GI cancers, improving disease- and progression-free survival when added to combination chemotherapy regimens. However, due to the intrinsic and acquired drug resistance, its clinical efficacy is limited, which is still a challenging problem for successful chemotherapy of hepatocellular carcinoma. Previously studies indicated that membrane transporter MRP2-mediated drug efflux effects determine the cellular accumulation and cytotoxicity of platinum drugs. Therefore, we hypothesized that the resistance effect could be reversed by perturbation of ABCC2 gene (encoding MRP2). The CRISPR-Cas 9 system, as a gene editing tool, can be used to knock out the gene of interest. MRP2 by manipulating genes. We hypothesized that the CRISPR-Cas9 technology can be applied for knocking-out ABCC2 in an in vitro cell model overexpression MRP2 (i.e. HepG2 cell line).

The HepG2 cells were transfected with ABCC2 guide-RNA/CAS9 protein ribonucleoprotein complexes through liposome-mediated delivery. The efficiency of ABCC2 gene disruption was then assessed using the T7 endonuclease I based method. The accumulation of a specific ABCC2 substrate 5(6)-carboxy-2',7'-dichlorofluorescein (CDCF) was determined in the gRNA/Cas9 transfected and wild type HepG2 cells by using flow cytometeric analysis. MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was undertaken to determine oxaliplatin sensitivity, and concentration-dependent cytotoxic effects of oxaliplatin was compared between gRNA/Cas9 transfected and wild type HepG2 cells.

Our current results reveal the feasibility of application of the liposome delivered CRISPR-Cas9 system to knock out ABCC2 gene in HepG2 cell line. Two sgRNAs were tested in this thesis, one of which showed that the target gene could not be

effectively knocked out for unknown reasons. Fortunately, the second gRNA plays a role in disabling target genes with a genomic cleavage efficiency of 25% in HepG2 cells. Increased model MRP2 substrate (CDCF) accumulation and oxaliplatin cytotoxicity was observed for ABCC2-knockout HepG2 cells compared to control wild type. These results demonstrate that the on-target genomic editing in cell line treated by CRISPR-Cas9, and silencing ABCC2 gene by CRISPR-Cas9 does reduced the function of MRP2 protein. This thesis provide prove-of principle in vitro evidence supporting a novel therapeutic strategy that knockout of ABCC2 gene can decrease MRP2 function and increase oxaliplatin chemosensitivity in hepatocellular carcinoma.

Acknowledgments

The thesis cannot be accomplished without kind support from my supervisor, my family, and friends. Therefore, I would like to express my heartfelt thanks to those people.

First of all, I would like to express my sincere and greatest gratitude to my primary supervisor Dr Yan Li for organizing such a fascinating research project for me to pursue this Master's degree. During this research journey, he patiently guided and gave me many thoughtful ideas and suggestion when difficulties encountered. It is because of his continued support throughout the project, encouragement and guidance, and his expertise in biomedical research that led me to complete this thesis successfully. So I would like to thank Yan for his patience and valuable supervision over the years, and let me explore the field of research and learn cutting-edge research skills from you.

I want to thank my second supervisor, Dr. Dong-Xu Liu, who is involved in the management of the laboratory and tries his best to improve research environment. He took the time to attend the meeting to share his expertise in research.

Also, I would like to thank everyone in the AUT Cell Culture Lab; Riya, Leo, Keven, Tinu, and Piyush who all have supported me, looked after me and helped me a lot not only in the lab work but also in the office works throughout this Master study. I am so lucky to get to know you all and work with you all who are very nice and kind to me.

Finally, I will not be able to start my first step in this great biomedical research graduate program and complete it without the love and care of my good parents, family and friends. Thank you very much, Mom and Dad are always by my side. During my years of study and through the research and writing of this paper, I have provided unremitting support and continuous encouragement. Without them, this achievement is impossible to achieve. Thank you.

Abbreviations

℃	Degree Celsius
5-FU	5-fluorouracil
ABC transporter	ATP-binding cassette
ANOVA	Analysis-of -variance
ATP	Adenosine-5’-triphosphate
bp	Base pair
CDCFDA	5(6)-carboxy-2,'7'-dichlorofluorescein diacetate
CDCF	5(6)-carboxy-2',7'-dichlorofluorescein
DACH	Diaminocyclohexane
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FBS	Foetal bovine serum
g	Gram
h	Hour
IC-50	Concentration at half-maximal inhibitory effect
kg	Kilo-gram
KO	Knockout
M	Molar concentration
µM	Micromolar concentration
mM	Millimolar concentration
µg	Micro-gram
mg	Milli-gram

µl	Micro-litres
ml	Milli-litres
mol	Mole
MDR	Multidrug resistance
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBD	Nucleotide binding domain
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
TMD	Transmembrane domain

Chapter 1 Introduction

1.1 Hepatocellular carcinoma

1.1.1 Cancer burden worldwide and in New Zealand

According to an earlier estimate in 2014, noncommunicable disease (NCDs) is responsible for about 38million death globally during 2012, and cancer was responsible for 21.7% amount them, which makes it the second most leading cause of NCDs death (Mendis, 2014). The latest statistic in 2018 estimates that there will be 18.1 million new cancer cases, with 9.6 million cancer deaths worldwide (Bray et al., 2018). Those statistics indicate that cancer has now ranked as one of the most deadly diseases, and as the main barrier to the increase of people's average life expectancy.

The estimate also reported that New Zealand has the world's worst rate of deaths from malignant skin melanoma, along with the worst rate of cases of leukaemia. The highest overall incidence finds both among men and women in Australia/New Zealand region (Bray et al., 2018). Colon, gastric, liver, pancreatic, rectal and other GI cancer types are a common cause of death and suffering in New Zealand. According to the most recent national cancer statistics, CRC and other GI cancers accounted for 4426 new diagnoses and 2747 deaths in NZ in 2013 alone.

1.1.2 Hepatocellular carcinoma status in New Zealand and worldwide

WHO's has approximated liver cancer as the fifth most commonly diagnosed cancer type and the fourth most leading cause of cancer mortality worldwide. In New Zealand, one of the most common types of cancer types is Liver or hepatic cancer. In 2011, liver cancer had cause 1.55% of newly diagnosed cases and 2.7% of total cancer mortality (Dae Won, Talati, Kim, & Kim, 2017). Furthermore, liver cancer incident was higher among Māori for all periods, compared with no- Māori (Chamberlain et al., 2013).

Primary liver cancer includes hepatocellular carcinoma (HCC) (comprising 75%-85% of cases), intrahepatic cholangiocarcinoma (comprising 10%- 15% of cases) as well as other rare types (Bray et al., 2018). HCC typically manifests in patients with previous liver damage. Those damages commonly caused by viral hepatitis (chronic hepatitis B and hepatitis C), metabolic disease (diabetes and nonalcoholic fatty liver disease), toxic agents (alcohol and aflatoxins), or immunoreacted disease (autoimmune hepatitis and primary biliary cirrhosis). However, compare to its well-defined etiology, there are no satisfactory therapeutic approaches to date (Alexopoulos, Melas, Chairakaki, Saez-Rodriguez, & Mitsos, 2010). Besides, only 15% of patients are eligible for potentially curative treatments. The rest majority of patients will present with advanced disease with poor overall treatment outcome and 5-year overall survival (OS) of 3-5% (Dae Won et al., 2017). Besides, the clinical treatment challenge of HCC depends not only on the stage of the tumour but also on hepatitis and cirrhosis, that related to liver function damage, both these symptoms can lead to poor tolerability to systemic chemotherapy (da Motta Girardi, Correa, Crosara Teixeira, & Dos Santos Fernandes, 2018).

1.1.3 Current chemotherapy regimens for hepatocellular carcinoma treatment

Chemotherapy is the type of treatment that uses anticancer drugs to help kill or stop the growth of tumour cells. These drugs can be administered orally or intravenously alone or in combination with other therapies. Some chemotherapy is given before the surgery to make the size of the tumour smaller or to make cancer more comfortable to be removed. Most drugs are applied to kill residual or unresectable tumors after surgery, as well as to prevent the disease from recurrence. Adjuvants chemotherapy used for advanced stage III/IV patients whose tumour is already too hard to be removed by surgery aimed to extend the date of survival.

Radical treatment, such as surgery and liver transplantation, is feasible for HCC at an early stage (Lane et al.). However, as previously mentioned, HCC could be triggered by multiple types of risk factors, including chronic viral hepatitis, excessive alcohol nonalcoholic fatty liver disease, or hereditary disease. Therefore, HCC often accompanied by a chronic liver or even cirrhosis that causes liver dysfunction (Grazie, Biagini, Tarocchi, Polvani, & Galli, 2017). Hepatic dysfunction, high heterogeneity, and recurrent diagnosis of advanced HCC have made this cancer hard to be treated directly by radical treatment as well as to develop new therapeutic alternatives (Grazie et al., 2017).

For targeted molecular chemotherapy, sorafenib was the first systemic therapy that demonstrates improved survival in patients with advanced HCC, and it for long also being the only standard drug. Sorafenib acts suppression of various molecular targets that are involved in the mechanism of tumor growth and angiogenesis. As a multi-kinase inhibitor that blocks vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) signalling, it is often used as a second-line treatment applied after fluoropyrimidine plus platinum-based chemotherapy. VEGF and PDGF were HCC proangiogenic cytokines that participate in the progress of HCC tumor growth (Sampat & O'Neil, 2013). The successful of sorafenib enlighten the investigation of new anticancer drugs that also target the alternative proangiogenic pathways, but most will eventually trigger the HCC resistance to anti-VEGF medicines drugs (Sampat & O'Neil, 2013). The side effect of doping sorafenib includes diarrhea, weight loss, hand/foot syndrome, and hypophosphatemia could happen (Grazie et al., 2017). Other molecular targeted therapy includes brivanib, a double tyrosine kinase, play as the inhibitor of growth factor receptor (VEGFR), and fibroblast growth factor receptor (FGFR) apply for clinical use. Those patients who intolerant with sorafenib

will alternatively take brivanib, but this drug shows a similar overall survival compared with sorafenib (Partridge, Watkins, & Mendenhall).

Sorafenib and brivanib have limited their usage on palliative care by their reduced efficacy on the treatment of advanced HCC. Other monotherapy regimens commonly apply for patients with the worse general condition or worse tolerance to drugs. Monotherapy includes doxorubicin, capecitabine, TS-1, FOLFOX4, GEMOX. Doxorubicin as the first chemotherapy drugs before the approval of sorafenib shows a 79% (11/14) of response rates at the initial phase II study but limited efficacy (<20% clinical response) in the following subsequent demonstrated with no significant survival benefit (Dae Won et al., 2017).

5-FU and capecitabine as single-agent chemotherapy have achieved decent clinical efficacy, and they were be used combined with other agents, including include cisplatin and oxaliplatin, for a better clinical outcome (Dae Won et al., 2017). 5-fluorouracil (5-FU) is nowadays one of the most widely used cancer medication, it can be applied for multiple types of tumours including colon, oesophageal, stomach and pancreatic cancer and has been evaluated for advanced HCC as well. A phase II study positively shows 28% response rates by using 5-FU combined with leucovorin in advanced HCC. But another phase II study of 5-FU with leucovorin showed inadequate clinical response with only I partial response (7%) (Dae Won et al., 2017). Capecitabine, which currently applied as adjuvant therapy after surgery, can convert to 5-fluorouracil (5-FU) and then slows tumour growth by acts on DNA synthesis. Compare with 5-FU; capecitabine shows a higher efficacy with better safety profile and good tolerability. TS-1 (Titanium-silicate) acts on the metabolism of 5-FU to increases its toxicity in neoplastic cells and shows a better result. However, TS-1 fails to prove its superiority results could be observed in a more specific population (Grazie et al., 2017).

Other combine treatments such as FOLFOX4 regimen (fluorouracil, leucovorin, oxaliplatin) have been evaluated as advanced HCC therapy. Despite the positive findings and an excellent safety profile, it shows no significant difference in terms of overall survival compare with doxorubicin. GEMOX regiment (gemcitabine, oxaliplatin) samely apply for the treatment of advanced HCC. The overall survival of this treatment shows to correlate with cirrhosis stage and tumour response rate to the drugs. GEMOX leads to better overall survival in comparison with a lack of response. The platinum-based anticancer agent is another common type of drug that applies for the treatment of HCC. Gemcitabine commonly employed for the treatment of pancreatic cancer. It could either be used as a single agent or in combination with erlotinib or capecitabine. Compared to standard gemcitabine treatment, FOLFIRINOX (oxaliplatin with 5-fluorouracil, folinic acid, and irinotecan) and GEMOXEL (gemcitabine, oxaliplatin, and capecitabine) both showed higher efficacy and better safety in patients with metastatic pancreatic cancer. Some oxaliplatin-based regimens such as XELOX (oxaliplatin plus capecitabine), GP (gemcitabine plus cisplatin) and cisplatin plus capecitabine have begun to be studied in phase || trials for HCC treatment and showing impressive results (Grazie et al., 2017).

1.1.4 Chemotherapy resistance in HCC

Also, the therapy and advanced screening recently have both reach some progress; the prognosis remains have no significant improvement. Multiple factors could affect the success of HCC treatment, include delayed of diagnosis. (Infantino et al., 2018). But the development of drug resistance is one of the primary reasons for the failure. The main barrier for successful HCC treatment includes chemoresistance mechanisms, not always useful chemotherapeutic, and delayed diagnosis. Sorafenib, as the only admitted drugs for HCC treatment, has a low response rate and eventually will trigger secondary

resistance not only for HCC but to all tumours types. However, the underlying acquired resistance to sorafenib are remaining unknown (da Motta Girardi, Correa, Crosara Teixeira, & Dos Santos Fernandes, 2018).

1.1.4.1 Heterogeneity in HCC

Cancer molecular heterogeneity can be defined as the distinct genetic alteration and phenotypes among cancer cells, found either between patients or within the same or multiple nodules originating from the same patient. The display of intra-tumor heterogeneity suggesting that the drug resistance clones may pre-exist within the same tumor nodule.

Molecular heterogeneity in cancer can be defined as the distinct genetic alteration and phenotypes among cancer cells, found either between patients or within the same or multiple nodules originating from the same patient. The develops of intratumor heterogeneity can either be the results of clonal evolution or cause by describes cancer stem cells (CSCs).

The primary clinical implication of intra-tumor heterogeneity is drug resistance, which shown to exist in the tumor before treatment. Therefore, when other cells die by targeted therapies, the resistant clones have room for proliferation. At this point, the dominant clone in the tumor is composed of the resistant clone and no longer respond to treatment. Additionally, it is believed that drug-resistant clones can evolve throughout therapy and become the predominant cone within a tumor (Villanueva, 2017).

1.1.4.2 Membrane transporter

Multidrug resistance (MDR) is another factor that limits the application of liver cancer chemotherapy, and it is also a significant cause of liver cancer recurrence and metastasis (Ceballos et al., 2018).

The well-recognized mechanism of MDR is the significant overexpression of adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of transporters, such as P-glycoprotein (P-gp/ABCB1), multidrug resistance-associated protein 1 (MRP1/ABCC1) and multidrug resistance-associated protein 2 (MRP2/ABCC2), et al., those transporter acts as an efflux pump on cell membrane and hence limit the overall drug intake (Chen et al., 2016).

To overcome MDR and improve the clinical outcome of HCC treatment. Some of the treatment strategies consists of inhibiting the activity or lower the expression of the efflux transporters.

1.1.4.3 Autophagy

Autophagy plays multiple roles in liver cancer in a different situation. In healthy liver cells, autophagy involved in the maintaining of liver homeostasis and preventing malignant tumorigenesis by removing harmful mitochondria and transformed liver cells. However, once HC is established, the autophagy acts as a tumor promotion role in tumor development, metastasis, and therapeutic resistance. In target HCC therapy, the role of autophagy is uncertain for either inhibiting or promotion, according to the different characteristic of agents. Studies have found that various chemotherapeutic drugs for HCC treatment can increase the autophagic flu of HCC cells, which may relate to the enhancement of drug resistance and the promotion of cell survival (Huang, Wang, & Wang, 2018).

1.2 Oxaliplatin

As previously mentioned, platinum-based drugs such as cisplatin and carboplatin have long applied in clinical as a treatment for various types of neoplasms patients. Oxaliplatin, also known as Eloxatin, is design as the third generation of platinum-based anticancer drugs. Compare with the first- and second generation, cisplatin, and

carboplatin, its structure has been enveloped to comprise by the platinum complex with [1,2-diaminoyclohexane (DACH)] ligand and oxalate as the leaving group. Those alternate on its structure gives some change to the characteristic of oxaliplatin compared to the previous two generations. First, oxalate leaves the group to reduce the reactivity of oxaliplatin and thus result in a reduction of side efficacy on human peripheral sensory neuropathy. Second, DECH could increase the lipophilic of the drugs and lead to the rise of overall drug uptakes. It also has been reported to increase the cellular entry pathway compare with the other two kinds of platinum-based anticancer drugs. Structure of oxaliplatin, cisplatin, and carboplatin showed in Figure 1.1.

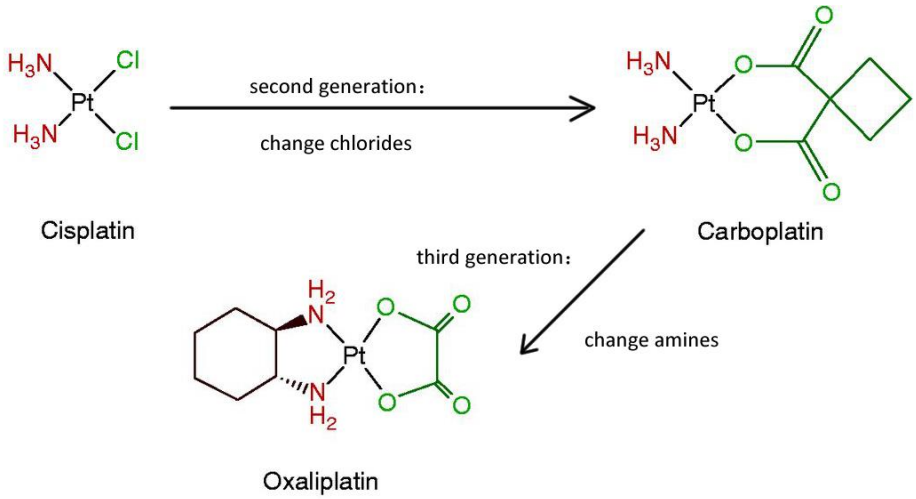

Figure 1. 1 The figure shows the structures of the first generation (cisplatinum), second-generation (carboplatin) and third-generation (oxaliplatin) of platinum, the carrier amine ligands in red and the labile chloride or carboxylate-based ligands in green. As prodrugs, all platinum-based drugs require the replacement of their labile ligands with water before they can bing and disrupt DNA function.

1.2.1 Clinical use

The chemotherapy drug oxaliplatin is critically important in the treatment of colorectal (CRC), HCC and other GI cancers, improving disease- and progression-free survival when added to combination chemotherapy regimens. The improved outcomes achieved with oxaliplatin are reflected in clinical practice guidelines, which recommend oxaliplatin-based chemotherapy as the standard preferred treatment for liver and other GI cancers (Grazie et al., 2017).However, its activity as single-agent oxaliplatin is

limited. Therefore, in most cases, oxaliplatin used in combination with other chemotherapeutic agents. For example, oxaliplatin can take with 5-FU, which in the past 50 years, being one of the most standard chemotherapy agents. Oxaliplatin and irinotecan (a topoisomerase ｜ inhibitor), and both can work in synergy with 5-FU/leucovorin treatment. The combined therapy can double the overall survival rate in advance disease (Jerremalm, Wallin, & Ehrsson, 2009). Also, oxaliplatin treated in combination with FP could improve its clinical outcome with an objective response rate of up to 50% in the first line and 15% in the second line. Other approved combination treatment strategies include FLOFOX (oxaliplatin, 5-fluorouracil with folinic acid), XELOX (oxaliplatin with capecitabine), and FOLFOXIRI (oxaliplatin, 5-fluorouracil, folinic acid, and irinotecan). Besides the application in the first and second line of cancer treatment, oxaliplatin-containing chemotherapy also used as adjuvant chemotherapy after surgical removal on locally advanced colorectal cancer. In the treatment of other diseases, such as the gastrointestinal, pancreatic cancer, gastric cancer and hepatocellular carcinoma, Oxaliplatin-containing chemotherapy act as an essential treatment strategy applied for patients.

1.2.2 Mechanism of action

Oxaliplatin belongs to the platinum-based antineoplastic family. Similar as other platinum-based anticancer drugs (cisplatin and carboplatin), oxaliplatin induces cytotoxic effects by formingplatinum-DNA adducts after displacing labile oxalate ligand. During this process, several transient molecules can be formed, including monoaquo a diaquo DACH platinum, which can covalently bindto macromolecules. Oxaliplatin is also able to cause several types of DNA cross-links,leading to DNA lesion and further blocking synthesis and transcription through binding with the guanine and cytosine moieties of DNA.

The mechanism of actions of oxaliplatin is shown in **Figure 1.2.**

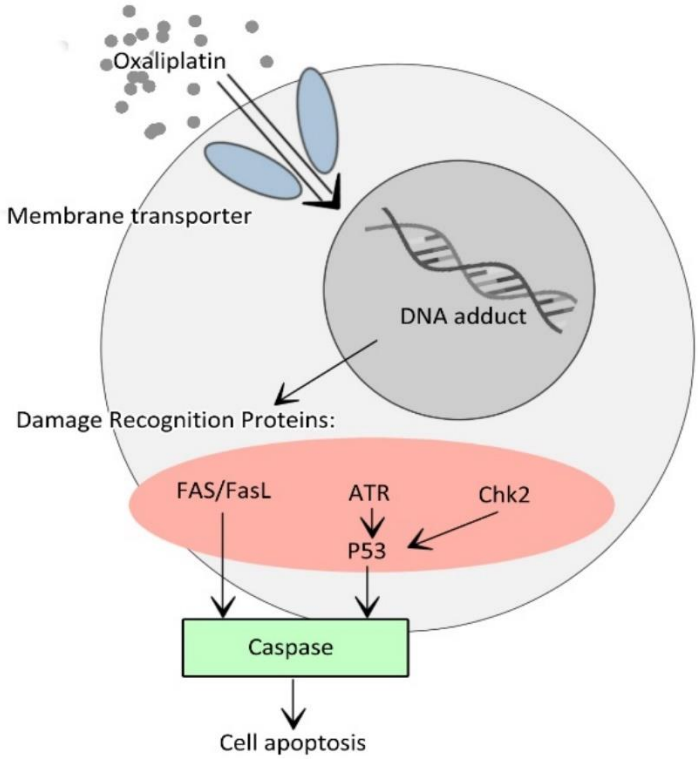

Figure 1. 2 Mechanism of actions of oxaliplatin. Platinum-based drug enters the cell through membrane transporters and forms DNA adducts in the nucleus, resulting in activation of DNA damage recognition proteins which then cause cell cycle arrest. Continuous exposure to platinum drugs further active the apoptotic machinery and lead to cell death.

Moreover, due to the differences in the mechanisms of inducing cellular cytotoxicity, oxaliplatin-DNA adducts cannot be recognized by mismatch repair (MMR) complex. MMR is a system that can detect cytotoxic adducts and appear as the main reason to induce cisplatin resistance. Therefore, cancer cells, with overly expressed of MMR, such as colorectal cancer cells, are not sensitive to cisplatin and carboplatin but will be substantially sensitive to oxaliplatin. This factor makes oxaliplatin being ideal treatment alternatives for those patients who have developed cisplatin- and carboplatin-resistant cancers.

Oxaliplatin-derived DACH-Pt-DNA adducts are bulky and more hydrophobic compared with cisplatin-derived cis-diamine-Pt-DNA adducts, which eventually causes a slower rate of Pt-DNA reactivity and makes oxaliplatin shows less amount of DNA lesions.

Even with a slower reaction rate, oxaliplatin presents more efficient DNA damage and cytotoxicity of oxaliplatin than cisplatin since oxaliplatin-derived adducts may contribute to the induction of immunogenic signals on the surface of cancer cells before apoptosis. Those signals can be active the interferon-gamma production and interaction with a toll-like receptor on the dendritic cells resulting in the immunogenic death of cancer cells. (Stein & Arnold, 2012).

1.2.3 Biotransformation and pharmacokinetics

After two hours of infusion, most of the platinum will be distributed to red cells and other tissues or eliminated from kidney. The oxaliplatin-derived platinum is irreversibly bound to plasma proteins such as albumin and gamma-globulins. In plasma, oxaliplatin undergoes rapid non-enzymatic biotransformation to form reactive intermediates (Jerremalm et al., 2009).

In this circumstance, oxaliplatin can react with a variety of molecules include water, chloride, glutathione, and methionine, as well as unidentified products and the biotransformation products, include Pt (DACH)Cl2, Pt (DACH)Cl (OH), Pt (DACH)(OH)2, Pt (DACH)(methionine), Pt (DACH) (glutathione). In vitro, oxaliplatin undergoes a slow reaction with DNA with the addition of its short half-life. It is suggested that the DNA adducts are formed by DNA with the biotransformation products of oxaliplatin (Jerremalm et al., 2009). Those biotransformation products have been proved to be more cytotoxic than oxaliplatin itself in a cellular assay, but this reaction can only be triggered when the platinum formed a ring-opened construction. After the ring-opened, oxaliplatin is then alternatively undergoing two pathways to form dihydrated oxaliplatin complex (DOC). First, it can react with bicarbonate or phosphate to hydrolyzed and give DOC. Second, it can react with the chloride to produce a dichloro complex which is subsequently hydrolyzed to DOC (Jerremalm et al., 2009).

In the first pathway, under a physiological condition, both bicarbonate and phosphate are less reactive with oxaliplatin. Therefore, only oxaliplatin could be biotransformed to more cytotoxic Pt (DACH)Cl2. Due to the lower accumulation, this complex does not contribute to the cytotoxic effect of oxaliplatin. Also owing to the slow conversion of oxaliplatin to Pt (DACH)Cl2, make this pathway less activation for forming DOC. In the other path, oxaliplatin could rapidly react with chloride to form a ring-opened structure intermediate, which then slowly transformed to Pt (DACH)Cl2. The process of oxaliplatin biotransformation is shown in figure 1.3

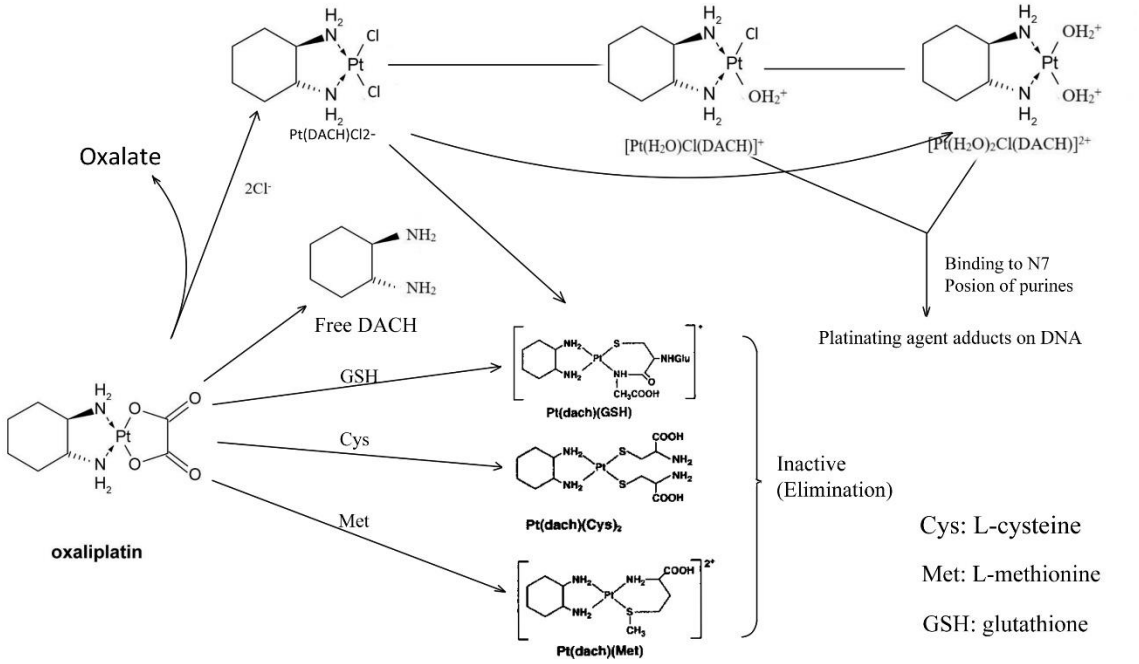

Figure 1. 3 Major biotransformation pathway of oxaliplatin.

Same as other platinum-based anticancer drugs, oxaliplatin is a high affinity towards sulfur-containing compounds, such as cysteine, methionine, and glutathione. It can form sulfur-platinum, which shows no activity in the bloodstream. Oxaliplatin also could react with γ-globulins, albumin, and hemoglobin, creating non-cytotoxic products (Jerremalm et al., 2009).

1.2.4 Clinical toxicities of oxaliplatin

The clinical toxicity induced by oxaliplatin includes moderate of myelotoxic, gastrointestinal, pulmonary, hepatotoxicity, and neurological.

Neurological toxicity is the most common dose-limiting side effect. Oxaliplatin may give two types of neuropathy: acute and chronic neuropathy. The occur of severe neuropathy is associated with the affection of the voltage-gated sodium channel. Acute neuropathy is reversible and primarily concentrates on peripheral nervous. It generally happens with hours or two days after dosing and able to resolves within 14 days (Han, Khwaounjoo, Kilfoyle, Hill, & McKeage, 2013).

In contrast, chronic neurology is a persistent neuropathy that may be associated with atrophy and mitochondrial dysfunction in the dorsal root ganglion cells caused by the accumulation of platinum compounds. It characterized by symptoms like paresthesia, dysesthesias, hypoesthesias, and deficits in proprioception which can interfere with daily activities. Chronic neuropathy occurs in 48% of patients receiving oxaliplatin and the symptoms can appear along without any priory acute neurological event.

Other clinical toxicities of oxaliplatin include pulmonary fibrosis, which affects less than 1% of patients. Hepatotoxicity, as evidenced in the adjuvant study, though the increase in transaminases and alkaline phosphatase, was observed more commonly in the combination arm of oxaliplatin.

1.2.5 Limitations of oxaliplatin-based chemotherapy

The oxaliplatin-based combination chemotherapy regiment can improve the overall survival time and disease-free survival time in multiple cancer types, but the maximal time was limited respectively to 19.5 months and nine months. The barrier for clinical application of oxaliplatin major on its limited efficacy and high toxicity in a wide proportion of treated patients. In a NZ observational patterns-of-care study, more than 40% of patients receiving chemotherapy including oxaliplatin stopped chemotherapy

early due to toxicity or lack of efficacy (Jackson *et al*, 2015).Pharmacokinetic parameters such as absorption, distribution, metabolism (biotransformation) and excretion variability as well as the intrinsic and acquired tumour resistance are obstacles to the bioavailability of oxaliplatin. On the other hand, as previously mentioned, side-effect such as the peripheral sensory neuropathy limiting the continues administration. Although limiting the dose can avoid the neurotoxicity caused by drug accumulation, the limited dosage also affects the toxicity of oxaliplatin to the tumour.

1.2.6 Tumour resistance to oxaliplatin

Both intrinsic and acquired resistance could happen during the treatment with oxaliplatin and contribution to the limitation on its efficacy. The reduction of overall drug intake and the increase of drug efflux are the primary factors to induce drug resistance. Other factors include the altered membrane permeability, detoxification of drugs with glutathione, and the alteration in DNA damage repair system also be considered associates with tumor chemoresistance.

The reduction of overall oxaliplatin cellular uptake or the increase of its cellular extrusion has declines the level of oxaliplatin accumulation, which further leads to a decrease in forming Pt-DNA adducts. This factor indicates that cellular transport is critical to the influence of the efficacy of oxaliplatin.

Another drug resistance mechanism is related to the inactivation or detoxification of oxaliplatin with glutathione. This effect leads to the formation of glutathione-oxaliplatin, which has been proved to have no cytotoxic effect on the tumor cell. Furthermore, the formation of platinum-DNA adducts can active the DNA damage repair system, which responsible for the detection and timely repairing of damaged DNA to protect the cells from death. The excision repair cross-complementation group 1 (ERCC1) is a crucial excision nuclease enzyme involved in nucleotide excision repair (NER). Pieces of evidence show that the increased activity of expression levels of

ERCC1is associated with the increase of oxaliplatin-containing drug resistance in virials types of cancer.

Alternation of cell apoptosis is another mechanism behind oxaliplatin resistance. Mutation in the apoptotic protein p53 and anti-apoptotic factors, including nuclear factor-κβ (NFκβ) and taxol-resistant gene 1 (Txr1), are involved in susceptibility changes induced by oxaliplatin. Tumour microenvironmental conditions, such as hypoxia, may also be included as the influence factor to the drug resistance mechanism of oxaliplatin.

Among all these mechanisms, this thesis focused on the membrane transport-related oxaliplatin efflux effect.

1.2.7 Cellular transport of oxaliplatin

There are Multiple factors could have an impact on cellular transporter efficiency of platinum-based anticancer drugs, those factors include membrane's stability, the affinity of substructure, type of platinum that involve in cancer cells, and the temperature.

Most of oxaliplatin cellular transport mechanism studies base on the previous research take on cisplatin. Cisplatin has been observed taking into cells through the lipid bilayer of the cellular membrane by passive diffusion. Thus, the increase of concentration and time cannot lead to saturating of the cisplatin, nor inhibit by its analogues. However, since cisplatin influx is an energy-dependent process, the overall cisplatin accumulation in tumor cells can be reduced.

Oxaliplatin can be the substrate of multiple membrane transporters include oxaliplatin includes organic cation transporter (OCTs) and copper influx transporter. Drug uptake mechanisms and drug export or efflux transporters include copper efflux transporter (ATP7A and ATP7B), multidrug and toxin extrusion transporters (MATE1 and MATE2) as well as ATP-binding cassette (ABC) transporter proteins (e.g. MRPs).

1.3 Membrane transporters

1.3.1 Overview of membrane transporters

The membrane transport system is a collection of transporter systems that responsible for regulating molecules to either influx or efflux through cells biological membrane, to help maintain the regular physiological activity of each living cell. Membrane transport proteins can transport a variety of critical molecular types, including proteins, glucose, minerals, organic ions, cellular metabolites, toxic substances, and xenobiotics. These molecules can pass through the lipid bilayer of the cellular membrane through a passive diffusion mechanism or carrier-mediated transport mechanism (Busch & Saier, 2002). Their transport mechanisms can classify membrane transporters as Channels/pores transporters, Electrochemical potential-driven transporters, primary active transporters, Group translocators, and Transmembrane electron carriers (Busch & Saier, 2002). Another classification base on the direction of the transport molecule. Under this classification, Membrane transporters can divide into uniport (one type of particles if carried in one direction), symport (two different kinds of molecules get through the membrane in the same direction), and antiport (two different types of molecules get through the membrane in exchange with each other) (Nies, A.T, 2014).

Primary pharmacology related transporters include the ATP-binding cassette (Robey et al, 2018;Adamska & Falasca, 2018) family, solute carrier (Drozdzik et al.) family, ion pumps and channels (Y. Huang & Sadee, 2006 n.d.).Some membrane transporters, such as ABC and SLC transporters, have a significant impact on Pharmacokinetics of multiple drugs, toxins, and endogenous compounds and their metabolites, drug disposition, drug resistance as well as physiological homeostasis to chemotherapy. Based on the influence of these transporters on drug intake, the drug resistances they trigger are one of the significant obstacles to the clinical improvement of anticancer drug efficacy.

On the other hand, more than a quarter of anticancer drugs are currently being developed as oral formulations to avoid costly and frequent hospitalization and potential infection risks (Oostendorp, Beijnen, & Schellens, 2009). However, efflux transporters expressed in the gastrointestinal tract (Adamska & Falasca) have been shown to limit oral bioavailability and absorption of oral formulation anticancer drugs.

Transporters expressed in the liver and kidney are decisive for drug disposal (Giacomini et al., 2010). Some of the therapeutic drugs which are substrates of the membrane transporters in the liver. Some medicines are taken up into hepatocytes, followed by metabolism while others are excreted into the bile in intact form. For example, CYP2C8 and 3A4 are responsible for the metabolism of cerivastatin after its hepatic intake (Yoshihisa, Toshiharu, & Yuichi, 2006).

Biotransporters located in the renal tubular epithelial cell membranes regulate the excretion of drugs gauging drug plasma concentrations, bioavailability, drug clearance, and drug excretion (Gomaa, Khan, Toledano, Waked, & Taylor-Robinson, 2008). Transporters located in other tissues, such as the blood-brain barrier, mammary glands, placenta, and testis-blood barrier, are responsible for determining the distribution of drugs in these tissues and controlling their toxicity to healthy tissues (Gomaa et al., 2008).

Transporters are also present on target cell membranes for controlling drug uptake and efflux of target cells, such as tumour cells. Thus, they can affect drug concentrations in target cells, drug-target interactions, and drug susceptibility and efficacy (da Motta Girardi et al., 2018).

Also, in addition to their physiological functions, membrane transporters are essential for pharmacokinetics (Bricca et al.), and pharmacodynamics (Adamska & Falasca) The drug disposition, drug efficacy, and sensitivity of the cell anticancer drug can all be affected in cancer pharmacology aspects by the membrane transporter. Among all

membrane transporters, ABC transporters as the transporter that majorly involved in the formation of cancer multidrug resistance (Adamska & Falasca) mechanisms been wildly studied (Y. Chen et al.).

1.3.2 Necessary ATP-binding cassette transporters in cancer pharmacology

The human ABC transporters family has 48 members that recognise a diverse range of substrate molecules and use energy from ATP hydrolysis to translocate them across membranes (Fletcher et al, 2010; Robey et al, 2018). ABC transporter export a wide range of endogenous ligands include amino acid, ions, peptides, vitamins, sugars, hormones, ions, lipids and xenobiotics (Vasiliou, Vasiliou, & Nebert Daniel, 2009). For example, signal lipid substrates include prostaglandin, leukotrienes, SIP, platelet-activating factor, cholesterol metabolites, cyclic nucleotides. Some small molecules include amino acids or sugars; macromolecules like polysaccharides or peptides can serve as substrates of ABC transporters (Hyde et al., 1990).

In addition to their role in maintaining physiological homeostasis, ABC transporters also play a fundamental role in tumour resistance, as well as defending the body against various drugs, toxins and carcinogens. The high expression in tumour tissues is associated with multidrug resistance because these proteins affect the overall drug accumulation by excreting cytotoxic or targeted anticancer drugs directly or indirectly from the cells (Fletcher et al, 2010; Robey et al, 2018). Although ABC transporters express in all types of the organs, they are significantly expressed in the kidneys, gastrointestinal tract, and liver (Adamska & Falasca, 2018), Therefore, they help control the efflux of xenobiotics, affect the absorption, distribution, elimination of the administered drug to determine bioavailability (Adamska & Falasca, 2018). ABC transporter increases drug efflux to reduce chemotherapy response.

ABC transporters expressed on the natural barrier of the gastrointestinal tract and basolateral membrane affect the bioavailability of the oral formulation (Adamska &

Falasca, 2018). The apical efflux transporter protein is mainly composed of ABC transporters such as ABCB1 and ABCC2. The overall drug intake can be affected since the drug that passes through the apical membrane can be efflux as a substrate for the apical efflux transporter back into the lumen (Lauretta M.S, Simon, & Barry H, 2004). These efflux actions, located in the first line of defense, act on maintaining regular physiological activity, limit the absorption of potentially toxic xenobiotics, and after the first extraction, the remaining drug components enter the systemic circulation through the liver or lymphatic vessels and are ultimately be discharged.

To date, 48 human genes and one pseudogene are responsible for encoding these kinds of protein members. The entire ABC transporters family can be divided into seven subfamilies (Robey et al, 2018), sharing a similar sequence and encoding a highly conserved structure. The structure contains four different domains. Two hydrophobic transmembrane domains (TMDs) pass through the putative α-helix and cross the membrane 5 or 6 times to form pores responsible for binding the substrate and mediating it through the lipid bilayer (Hyde et al., 1990). The other two domains are coupled to ATP hydrolysis. Proteins utilize a gradual change in the energy that is hydrolyzed from ATP to its domains. The substrate molecule is then allowed to transport against its concentration gradient across the plasma and intracellular membrane leading to diminished substrate accumulation. These four domains can merge in different ways. In some bacterial systems, the four domains encode separate polypeptides and require periplasmic substrate-binding protein activity, and some ribose ATPs can bind to the single fused polypeptide (Hyde et al., 1990) the main fragment of ABC transporter structure shows in the figure below.

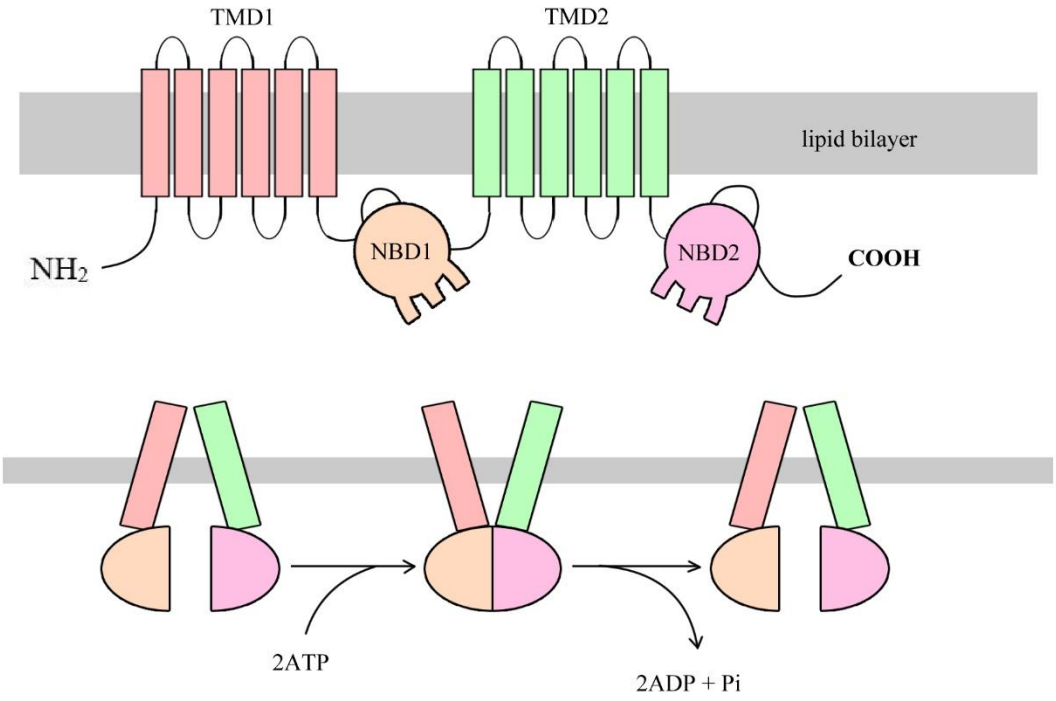

Figure 1. 4 Primary structure and the ATP-switch mechanism of ABC transporter

The expression of the ABC transporter has influences on the efficacy of the antitumor drug. In normal physiology, ABC transporters are involved in drug absorption, distribution, and elimination to determine the bioavailability of the administered drug. However, the effect of ABC transporters needs to be achieved by drug penetration. Therefore, the expression of ABC transporters may affect the pharmacokinetic profile of the administered chemotherapeutic agents (Adamska & Falasca, 2018). Screening with the NCI60 cell panel indicated that up to 31 of the 48 ABC transporters attenuated the efficacy of the anti-tumor drugs screened in the study. Besides, many other transporters not associated with the ABC family may play a role in drug sensitivity and disposal (Szakács et al., 2006).

In addition to being a drug pump, ABC transporters also affect tumor progression. Many ABC transporters are involved in the secretion of biologically active molecules, and the transport of signaling lipids, such as fatty acids, cholesterol, peptides, sterols and xenobiotics, and have been shown to contribute to cancer progression. For example,

ABCA1 is involved in reverse cholesterol transport and phospholipid transport to the plasma membrane. Recent studies have shown that ABCC1 participates in the ovary by extruding lipids (lysophosphatidylinositol, sphingosine-1-phosphate) that play an essential role in carcinogenesis progress in ovarian cancer and prostate cancer. Wildly evidence believes that ABC transporter activity is involved in cancer cell proliferation, migration, invasion, and anti-apoptosis (Fletch et al, 2010). These factors have led to the study of ABC transporters not limited to their role in drug resistance but their correlation to cancer development, progression, and invasiveness (Adamska & Falasca, 2018). Studies have shown that the expression levels of ABC transporters in healthy stem cells and cancer stem cells are relatively high. However, cancer stem cells can reduce tumor differentiation and also result in higher proliferative potential and a higher invasive phenotype, while higher ABCC transporter expression levels are found in tumor subtypes or regions of lower differentiation. ABCB1 was also found to be expressed in different colon tumors, but there was no similar situation in healthy colon tissue. Also, ABCG2 expression levels were found to be linked with tumorigenic proliferation, drug resistance, and metastatic ability.

Several strategies have been used to inhibit the expression of ABC transporters in tumour cells. These strategies include inhibiting the activity of transporters, preventing transcription factors from regulating their expression, and blocking transporter-induced signalling pathways. To date, three generations of modulator inhibitors have been invented, the first generation including verapamil, quinine, or cyclosporin A, which show significant toxicity. Valspodar (cyclosporin A derivative), a candidate for a second generation ABCB1 inhibitor, showed unsatisfactory results in clinical trials, although increasing its efficacy and reducing toxicity. All of these limitations result in the development of third generation or inhibitors. However, the lack of significant positive and off-site effects has stopped most clinical trials (Adamska & Falasca, 2018).

Several factors could influence the efficiency of the inhibitor. First, high dosage and off-target action could lead to higher toxicity in healthy tissues. Therefore, the overall dosage has to be limited. Second, some of the ABC transporter substrates, such as CYP450, are involved in drug metabolism. Their interaction affects the pharmacokinetic properties of co-administered chemotherapeutic agents, which lead to the change of activity and further to reduce the drug efficacy and moreover to increase the toxicity. Third, Evidence shows there is a strong correlation between the expression of several ABC transporters (Szakács et al., 2006). Due to the unspecific blockade, the case of using the separation of anticancer agents alone with natural xenobiotics is reduced. However, utilizing multiple inhibitors may also lead to unsatisfied results. The inhibition on one transporter might result in a compensated by the upregulation of another (Adamska & Falasca, 2018).

1.3.2.1 ABCB1

ABCB1 (P-glycoprotein/MDR1/P-gp) mainly distributes on the apical surface of excretory and epithelial cells. It is expressed in cells of the gastrointestinal tract, small intestinal membrane, blood-brain barrier, hepatocytes, kidney, pancreas, and brain adrenal gland to preventing toxins xenobiotics from entering the nervous system (De Vera et al., 2019). The enriches of P-gp in the oral, intestinal barriers indicate its essential role in the absorption of the orally administered formulation. P-gp is related to the transportation of more than 200 of diversity molecules (Adamska & Falasca, 2018). The enriches of P-gp in the oral, intestinal barriers indicate its essential role in the absorption of orally administered compounds. P-gp is involved in the transport of more than 200 molecules (Adamska & Falasca, 2018), and is responsible for the development of tolerance of cells to neutral and cationic hydrophobic compounds.

As a member of the ABC transport family, It shares a similar structure with other members. Its structure comprises two homologous nucleotide-binding domains, and two transmembrane domains joined by a linker region. P-gp has a specific tendency toward lipophilic, cationic compounds, and various hydrophobic molecules (Lauretta M.S et al., 2004). The substrates of P-gp transporter include anticancer agents, antibiotics, antiviral agents, calcium channel blockers, and immunosuppressive agents. It involves the efflux of many types of anticancer drugs, such as anthracycline (doxorubicin, daunorubicin), epipodophyllotoxin (epoxide, teniposide), taxanes (paclitaxel, docetaxel), vinca alkaloids (vincristine, vinblastine), tyrosine kinase inhibitors of EGFR, VEGFR and Bcr-Abl, anthracycline, HIV-protease inhibitor calcium channel blockers, steroid hormone antibiotics, lipids.

Over the years, various strategies have been applied to deal with drug efflux effects caused by P-gp overexpression. The proposed combination treatment（using two modulator anticancer agents） might be a promising strategy for MDR reversal. Combination therapy can reduce the overall concentration of the modulator and therefore, to reduce the side effect. Aclarubicin (ACL) found less P-gp dependent than doxorubicin (DOX). The study shows the reactive oxygen species (ROS) and reduces the expression of P-gp. Immune-oncology agent IPI-549 is the modulator of P-gp. Selonsertib, which used to be seen as an ASK1 inhibitor, is first found able to stimulate the activity of ATPase for both P-up and ABCG2. Therefore, it can suppress the expression of ABCB1 and ABCG2. (Ji et al., 2019).

Midostaurin is an orally bioavailable multikinase inhibitor, which can inhibit the activity of both angiogenesis and protein kinase C(PKC). It found significant antiproliferative activity in human cancer cell lines and murine xenograft models. By increase drug-induced apoptosis and triggered significant inhibition of ATPase activity

of P-gp, midostaurin can treat newly diagnosed FMS-like tyrosine kinase-3 (FLT3)-mutated AML and advanced systemic mastocytosis (SM). This drug can selectively inhibit the activity of transport by reverses multidrug resistance in ABCB1-overexpressing cancer cells at non-toxic nanomolar concentrations (Hsiao et al., 2019). Other new findings may inspire the new inhibit strategy. Such as the Aclarubicin (ACL) found less P-gp dependent than doxorubicin (DOX)., thus, it may be used as the alternative drug to avoid the resistance. There is also a study found that the reactive oxygen species (ROS) can cause the reduces the expression of P-gp.

Some molecular are found relative to the expression of P-gp. Recent studies indicated that various cytokines such as vascular endothelial growth factor (VEGF) and interleukins (ILs) are involved in the pathogenesis of osteosarcoma. ILs interleukins, especially IL-8, are thought associated with the increase of P-gp transcription, it able to up regular promoter activity while not affect protein or mRNA stability (Cheng et al., 2018).

Cancer stem cells (CSC) found strongly associated with chemotherapy resistance and postoperative recurrence. It correlates to the formation and progression of cancer. CSCs are cancer cells that possess characteristics associated with healthy stem cells. The generate tumors through the stem cell processes of self-renewal and differentiation into multiple cell types. Some of the ABC transporters include ABCB1, ABCG2 and CD133 are the markers of CSCs.

1.3.2.2 ABCCs (MRPs)

ABCC family contains the highest number of drug transporters with 9 out of 12 members involved in drug resistance mechanisms (Adamska & Falasca, 2018). Members of the ABCC subfamily can transport organic anions and phase II metabolites, such as cystic fibrosis. Transmembrane conductance regulators, the only

members of the ABC family that act as channels, play an essential role in chloride ion efflux (Fletcher et al., 2010). The synergy between the efflux system and the metabolic/conjugating enzyme provides a powerful alliance for drug elimination. In addition to the MDR-like core structure consisting of two NBDs and two TMDs, the ABCC subfamily also includes other domains. ABCC1, ABCC2, ABCC3, ABCC6, and ABCC10 contain an amino (N)-terminal membrane-bound region connected to the core by a cytoplasmic linker. The remaining four members (ABCC4, ABCC5, ABCC11, and ABCC12) lack the N-terminal TMD (but there is also no linker region, which is characteristic of this subfamily) (Szakács et al., 2006).

1.3.2.2.1 MRP1

ABCC1(MRP1) expressed in a wide range of tissues, clinical tumors, and cancer cell lines including breast, lung, ovarian and prostate, and correlates to the poor chemotherapy treatment outcome. The expression of ABCC1 confers resistance to methotrexate, vinca alkaloids, anthracycline, and camptothecin, thereby affecting the therapeutic effects of various anticancer drugs (Adamska & Falasca, 2018). It involves in the resistance to several hydrophobic compounds, which also are the substrates of P-gp. The structure of ABCC1 is similar to other members of the ABCC subfamily. ABCC1 can export glutathione (GSH), glucuronate or sulfate conjugates of organic anions. Its homologs implicated in the resistance to anticancer agents include ABCC2 (MRP2), ABCC3 (MRP3), ABCC6 (MRP6) and ABCC10 (MRP7) (Szakács et al., 2006).

1.3.2.2.2 MRP2

Unlike most of the ABCCs subfamily members expressed on the basolateral membranes, ABCC2 (MRP2) localized on the apical membranes of polarized cells typically found in hepatocytes and enterocytes. It has a pivotal role in the export of

organic anions, unconjugated bile acids, and xenobiotic into the bile, also play a part in the control for the absorbance of orally ingested drugs. Mutations occur in the gene which encodes MRP2 can lead to a lack of hepatobiliary transport of non-bile salt organic anions and additionally results in conjugated hyperbilirubinemia, which called Dubin-Johnson syndrome. Some substrates of MRP2 are the same with MRP1, such as the gemcitabine, which is a wild used nucleoside analogue effectively in the treatment against numbers of cancers. Gemcitabine loaded conjugates exhibited electrostatic-interaction with the intestinal epithelial cells, and both P-gp and MRP2 could efflux affected the cellular transport of the conjugated(Chen et al., 2018). Cells that treat with cisplatin, arsenite, or 9-nitro-camptothecin, will observe an increased expression of MRP2. Clinical cancer specimens of renal, gastric, colorectal have found MRP2 can predictive chemotherapy response(Szakács et al., 2006). Intestinal MRP2 is active in regulating the pharmacokinetics, and consequently, the therapeutic efficacy of orally-administrated therapeutic agents. The restriction imposed on intestinal drug absorption by MRP2 is part of the universal feature of ABC transporters, known as multidrug resistance (MDR). Several strategies have been developed to revert MDR, like the use of protein inhibitors, monoclonal antibodies, and antisense oligonucleotides or interfering RNA. MDR reversal of specific MRP2 has not been extensively studied because most MRP2 substrates can also be transported by other ABC members, which makes this treatment ineffective.

Regarding strategies directed to overcome the restriction imposed by intestinal ABC transporters towards drug absorption, the few studies available are mainly directed to MDR1 (Arana, Tocchetti, Rigalli, Mottino, & Villanueva, 2016)

1.3.2.3 BCRP (ABCG-2)

ABCG2(MXR/BCRP) is a high-capacity transporter with broad substrate specificity. The list of ABCG2 substrates is vast and diverse, include cytotoxic drugs, toxins, and carcinogens found in food products, as well as endogenous compounds. Its expression associated with the resistance to doxorubicin, mitoxantrone, anthracyclines, and topotecan (quinolone topoisomerase inhibitor). Due to its diversity of substrate specificity and high expression in the intestine kidney and liver, ABCG2 plays a vital role in the absorption, distribution, and excretion of many prescription drugs. While newly developed drugs are now routinely found as the substrates/inhibitors of ABCG2, many older medications, including many statins, are also the substrates of ABCG2. The variation in the expression and activity of ABCG2 can have a significant impact on the efficacy and safety of these medications (Brackman & Giacomini, 2018).

ABCG2 transporter is mainly expressed in ovaries, brain, liver, prostate, placenta, and small intestine and plays an essential role in the development of various cancers. The essential part of ABCG2 for the transport of physiological substrates is remaining elusive. It has been suggested the function of ABCG2 is associated with protecting cells from various noxae by extrusion of harmful substances from intracellular. This conjecture is supported by the high expression of the ABCG2 level in the liver, in tissue barriers and stem cells (de Boussac et al., 2012). For example, in some sensitive tissue, such as the placenta and brain, ABCG2 has been observed in a higher level of expression in the blood-brain barrier aim to protect the brain from toxic pharmaceuticals. Some of the endogenous compounds, such as porphyrins, can be toxic at high levels and under hypoxic conditions, will be the substrate of ABCG2. Besides, There has been reported an increase of ABCG2 expression in pluripotent stem cells, suggesting its role in the maintenance and protection of stem cells.

Similar to its functions in healthy cells, highly expressed ABCG2 in types of tumor cells is associated with their development to drug resistance. In cancer, such as acute myeloid leukemia, non-small cell lung cancer, ovarian cancer, chronic myeloid leukemia, and esophageal squamous cell carcinoma, the tumor expression level of ABCG2 was found to have a significant association with survival and response to therapy, typically, a higher ABCG2 expression level will link to a worse prognosis. In a panel of 150 untreated solid tumors, ABCG2 expression was evident in all of them, but exceptionally high in cancers of the digestive tract endometrium, and lung and in melanoma (Brackman & Giacomini, 2018).

1.4 Role of membrane transporters in oxaliplatin uptake and resistance

A variety of biological mechanisms have been identified to be involved in the development of tumor resistance against platinum-based drugs. Among them, the ABC family, as membrane transporters, plays an essential role in the progress of chemical resistance by pumping out chemotherapeutic agents (Zhang et al., 2018; Myint et al., 2019).

1.4.1 Copper transporters

Copper influx and efflux play a vital role in the accumulation of platinum-based drugs. For example, human copper transporter 1 (hCTR1) is involved in the uptake of oxaliplatin. Studies have shown that the up-regulation of hCTR1 can control the production of resistance to cisplatin and carboplatin, although this effect is reduced in the case of oxaliplatin. Besides, two intracellular types of ATPase, ATP7A, and ATP7B, which are involved in the sequestration and extrusion of copper, have also been shown to be resistant to platinum drugs. STP7A and STP7B have also been found to be capable of sequester of cisplatin, carboplatin, and oxaliplatin into subcellular compartments to limit the cytotoxicity of platinum drugs.

Furthermore, proficient transporter cells are resistant to triggering the transport of STP7A to the plasma membrane, which appears to be critical for its ability to export copper from cells. In the case of oxaliplatin, this fact is associated with an increase in the level of platinum adducts in the DNA, but not in cells treated with cisplatin or carboplatin (Martinez-Balibrea et al., 2015).

1.4.2 Solute carrier superfamily of membrane transporters

Solute carrier (SLC) subfamilies are currently found contain 55 transporters members and play a role in the physiological uptake and/or excretion of drugs and xenobiotics in the intestine, liver, and kidney. Among them, the organic cation transporter (OCT) subgroup consisting of SLC22A1 (OCT1), SLC22A2 (OCT2) and SLC22A3 (OCT3) is involved in the transport of platinum-based drugs. Mainly, OCT2 was found to be significantly associated with the uptake of cisplatin and oxaliplatin and its cytotoxicity (Martinez-Balibrea et al., 2015). However, in analysis of a clinical gene expression dataset, ABCC2 (encoding multidrug resistance-associated protein 2, MRP2) was the only one of 18 oxaliplatin transporter candidate genes with differential tumour expression between CRC patients who did or did not respond to oxaliplatin-based chemotherapy (Myint et al., 2019).

1.4.3 ABC transporters

The ABC transporters are reported to be responsible for the efflux of more than 80% of the current chemotherapeutic drug. The ABCC subfamily containing multidrug resistance-associated protein (MRP) is associated with the development of resistance to platinum-based drugs. As examples, the expression of MRP1 and MRP4 associated with oxaliplatin resistance is increased, and changes in N-linked glycosylation of these transporters are associated with decreased drug accumulation in ovarian cancer in vitro models and increased oxaliplatin resistance (Martinez-Balibrea et al., 2015).

MRP2 is an integral 190 kDa protein, encoded by the ABCC2 gene and is expressed in the canalicular membrane of hepatocytes, luminal surface of renal proximal tubules, and in the small intestine, placenta, and brain where it functions as a gatekeeper to extrude endogenous and exogenous compounds. MRP2 plays a major role in the body's defence against drugs and toxins by controlling their oral bioavailability and distribution in healthy body tissues, and by facilitating their excretion into bile and urine (Bugde et al., 2017; Robey et al., 2018).

Patients with Dubin–Johnson syndrome, where mutation of MRP2 causing functional deficiency in humans, have developed hyperbilirubinemia. Many different approaches have been used in order to identify substrate transported by MRP2. Differential analysis of drug uptake into inside-out membrane vesicles derived from normal and MRP2-overexpressing cells (Myint et al., 2015) as well as in cell lines transfected with human MRP2 have determined the accumulation of substrates in cells (Myint et al., 2019). In membrane vesicles MRP2 transports oxaliplatin in a ATP-dependant manner with Km of 301 μM and Vmax of 2680 pmol Pt/mg protein/10 minutes) (Myint et al., 2015)

Apart from oxaliplatin, MRP2 also transports various drugs including ceftriaxone, ampicillin methotrexate, vinblastine, irinotecan, pravastatin and cisplatin (Bugde et al., 2017). In addition, MRP2 is the major ABC transporter responsible for the active transport of glucuronide- and sulfate-conjugated drug metabolites. MRP2 (ABCC2) is overexpressed on the plasma membrane of several CRC lung, gastric, and hepatocellular carcinoma cell lines and higher ABCC2 gene expression is detected in early CRC tissues compared to non-tumorigenic tissues (Hinoshita, et al., 2000; Szakács, et al., 2004). Inhibition of MRP2 by a model inhibitor such as cyclosporine-A, benzbromarone, bromosulfalein and MK571, can increase drug or substrate accumulation in the tested cells and/or a decrease their biliary excretion. Some of the

phytochemical inhibitors like curcumin and myricetin also inhibit MRP2 expression/function, which increased oxaliplatin accumulation and sensitivity in GI cancer cells (Myint et al., 2019) and decreased hepatobiliary excretion of drugs and their metabolites. However, the off-target effects cannot be fully ruled out, given the multi-target effects of these phytochemicals.The drug efflux through the glutathione (GSH)-mediated, which is in turn mediated by the ABCC family transporters, related to the decrease in the accumulation of intracellular platinum-based drugs, including oxaliplatin. Once the drugs are reaching the cytoplasm, oxaliplatin becomes hydrated, which promoting its reaction with sulfhydryl-containing molecules such as GSH or metallothionein. This reaction is considered as an essential resistance mechanism since there is evidence that shows the association between the levels/ activity of GSH and oxaliplatin resistance in vitro (Martinez-Balibrea et al., 2015).

1.5 CRISPR-Cas9

1.5.1 Mechanism of the CRISPR-Cas9 system

CRISPR-Cas9 is derived from the prokaryotic immune system of bacteria. As part of the defending device, CRISPR-Cas9 is responsible for the disruption of invading foreign plasmid or viruses (Saber, Liu, Ebrahimi, & Haisma, 2019). Since CRISPR-Cas9 can activate site-specific DNA double-strand breaks (DSBs), it is nowadays regarded as a promising gene-editing tool (Ghosh, Venkataramani, Nandi, & Bhattacharjee, 2019).

CRISPR-Cas9 system consists of two essential components: a single-guide RNA (sgRNA) and Cas9 nuclease. Cas9 usually derived from *Streptococcus pyogenes*, it can cleave double-stranded DNA. Cas9 protein contributes by two nuclease domains: HNH and RuvC-like. HNA can cut the complementary DNA strand, whereas the RuvC-like domain can cut the non-complementary strand (Saber et al., 2019). SgRNA is a 20bp nucleotide designed to pair the target DNA sequence based on Watson-Crick base

pairing (Xia et al., 2019). SgRNA sequence decided the accuracy of CRISPR-Cas9 genome editing since the first 10-12 nucleotides at the 3' end of gRNA worked on recognizing and binding the target sequence (Liu, Saber, & Haisma, 2019). Once the sgRNA links to the target site, it directs the sgRNA/Cas9 complex located as approximately-3 nucleotides in front if adjacent protospacer motif (PAM) towards targeted sequence (Saber et al., 2019). This reaction then induces a conformation change of the Cas9 protein, two nuclease domains formed to cleaved both strands of the target DNA, lead to the generation of DSBs. SpCas9, a widely used Cas9, can recognize most of the common NGG PAMs (Kleinstiver et al., 2015). It is frequently occurring in the genome and has less restriction on the selection of the target site (Xia et al., 2019). Two nuclease domains of Cas9 protein worked with limited correlation. Mutations that happened in single amino acid will lead to a single-stranded DNA nick instead of DSBs. Cas9 will lose its ability to cleave DNA if both nuclease domains have mutated (Xia et al., 2019).

Once the DSBs formed in mammalian cells, two major repair pathways are immediately evolved to repair it: non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Ghosh et al., 2019).

NHEJ is the primary pathway, and it repairs DSBs by chemically ligated back the two ends together (Baliou et al., 2018). This pathway is efficient but usually results in small insertions or deletions that occurred at the breaking site (Xia et al., 2019). Those insertions/deletions can cause frameshifts or premature stop codons, which further lead to the disruption of the target gene (Karimian et al., 2019).

In contrast, the HDR pathway is less efficient but more accurate. In this gene repair mechanism, a DNA template searching for a homology position at the flanking site of the DSB to reactivity the dysfunctional genes and restore gene function. In the CRISPR system, this pathway is utilized for silent mutation, which anticipatedly introduces

single-strand DNA oligonucleotides to the target gene and phenotype in a particular cell type (Xia et al., 2019).

The phase of the cell cycle affects the selection of the repair pathway. NHEJ common happened when the cell at G1, S or G2 phases, whereas, HDR could only employ to the cell at S and G2 periods (Baliou et al., 2018).

Those two pathways are employed differently in genomic editing based on their repair mechanism. NHEJ pathway usually offered for gene disruption (include causing small deletions or insertion), inversions, duplications or deletions. On the other hand, since HDR mechanism can introduce new genetic information into the genome, it is usually be applied for deletions, base mutations, insertions and replacements (Baliou et al., 2018). A brief overview of CRISPR Cas9 system and repair mechanisms is shown in Figure1.4.

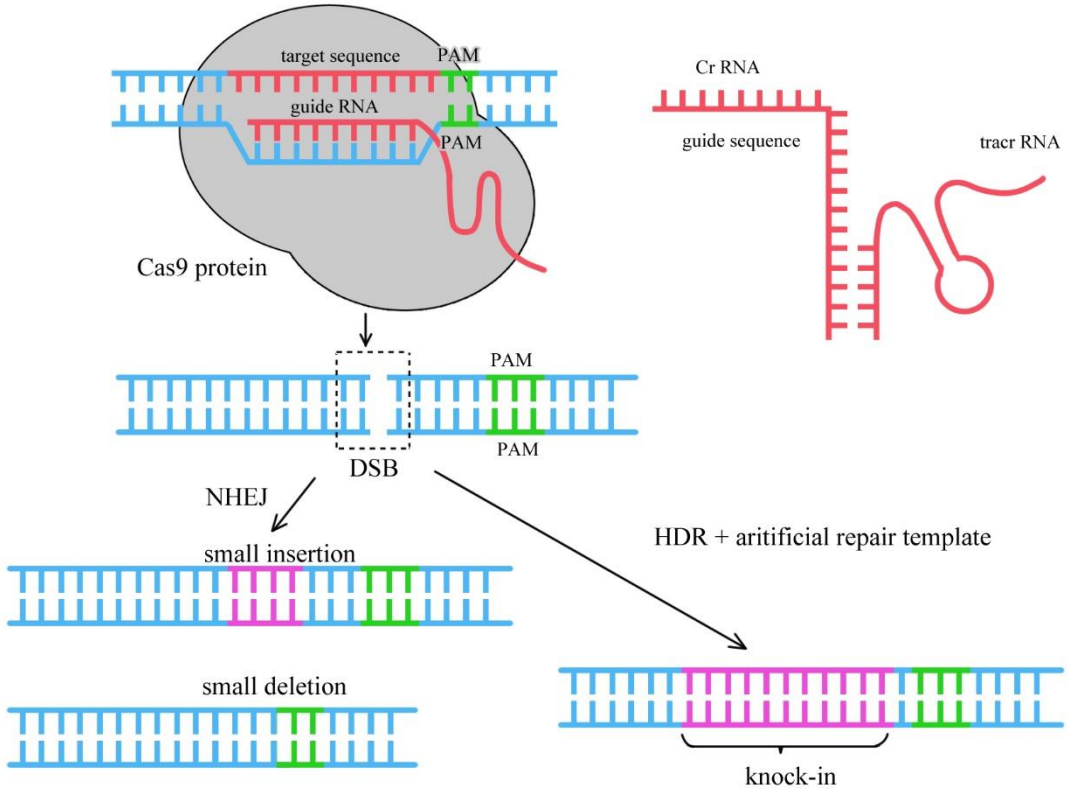

Figure 1. 5 The mechinasm of CRISPR/Cas9 system

1.5.2 Application strategy in cancer therapeutics

Cancer is a multistep process that involves the accumulation of genic changes. Those changes include mutations, genome rearrangements, and epigenetic alterations that occurred in oncogenes and tumour suppressor genes. It is essential to investigate normal and cancerous cell genomes to investigate the initiations process and study the therapeutic response of cancer cells for treatment development. (Rodriguez-Rodriguez, Ramirez-Solis, Garza-Elizondo, Garza-Rodriguez, & Barrera-Saldana, 2019). Thus, CRISPR-Cas9 technology, as a gene-editing tool, have broad potential applications in this area. It could either be applied to identify the possible molecular mechanisms of tumour growth or directly participated in a variety of therapy methods (Baliou et al., 2018).

1.5.2.1 CRISPR screen applied in anticancer drug development

Molecularly targeted drugs have been widely used in a variety of cancers. However, not all cancer types have found potential therapeutic targets, and even molecularly targeted drugs are facing the problem of drug resistance development. It is essential to identify molecular targets and elucidate resistance mechanisms to overcome all types of cancers. Random mutagenesis screens, such as retroviruses, transposons, and shRNA libraries, have been used to discover new drug target genes on tumour cells (Kurata, Yamamoto, Moriarity, Kitagawa, & Largaespada, 2018). Since CRISPR-Cas9 is a powerful editable RNA-directed nuclease that has been used for precise genome editing in a variety of model systems. Large CRISPR libraries are manipulated to discover specific genes and genetic elements involved in biological processes and disease-related phenotypes (Guo, Chitale, & Sanjana, 2017). Such loss of function libraries appears to be robust and efficient for identification of novel mechanisms such as drug resistance and cell survival signals (Kurata et al., 2018). In addition to being initially applied to the loss of function,

the combined CRISPR screen is also applicable to gene overexpression, inhibition, and enhancer subdomain regulation (Guo et al., 2017).

There are mainly three different CRISPR screen platforms applied for various aspects. a) CRISPRn: the gene knockout by CRISPR/Cas9 introducing indel mutation by creating DSBs on the targeted coding exons . b) CRISPRi: CRISPR interference is a genetic perturbation technique that allows sequence-specific gene repression when targeting near the promoter. This is achieved by combiningthe Krüppel associated box (KRAB) repressive element with a catalytically inactive form of Cas9. . c) CRISPRa: CRISPR activation using direct fusion or recruitment of transcriptional activation domains such as VP64, p65, HSF1, Rta to activates gene transcription at the endogenous locus (Chavez et al., 2016; Joung, et al., 2017). CRISPRa simply requires the synthesis and cloning of RNA guides, making it much more affordable to overexpress gene of interest within endogenous regulation (Guo et al., 2017; Joung et al., 2017).

For drug development applications, CRISPR technology can be used to identify new promising therapeutic genomic targets to avoid resistance. It can also be used to understand critical genes that drive to the drug resistance to improve the efficacy of current anticancer drugs. It can provide alternative targeting options. When identifying noncoding regulatory elements that affect oncogene expression or the oncogene itself cannot be treated with medications (Guo et al., 2017).

Thus, in general, the primary usage strategy for CRISPR screening can be the following categories: 1). Understanding synthetic lethality and identifying potential new therapeutic targets through screening for cancer- and stage-specific dependencies; 2) identifying genes that confer resistance or sensitivity to exist targeted therapies; 3) targeting noncoding regulatory areas that influence oncogene expression to provide

therapeutic alternative in cases where the oncogene itself may not be druggable (Guo et al., 2017).

1.5.2.1.1 Identifying cancer-specific vulnerabilities

Due to the possibility of mutational processes and differences in genomic instability, cancer cells often advance different genomic features during cancer development. The identification of which mutant protein or gene expression programs lead to tumor proliferation can identify cancer-specific vulnerability. For example, Using CRISPR-Cas9 to investigate the influence of GPRC5a on the proliferation and migration of PaCa cells as well as its effects on chemotherapy drug resistance, and the result indicates that GPRC5a was upregulated in PaCa tissues and various PaCa cell lines. Furthermore, knockout out of GPRC5a, reduced the proliferation and migration ability of PaCa cell lines and enhanced sensitivity to gemcitabine, oxaliplatin, and 5-fluorouracil in PaCa cells. Those results promote the development of PaCa targeted therapy (Liu, Yang, Pilarsky, & Weber, 2018).

With the utilize of CRISPR-Cas9 technology, much of the efforts have been put on the identification of new potential therapeutic targets that may further applied in clinical (Guo et al., 2017). By apply CRISPR-Cas9 to different cell lines, it is resulting in the identification of shared essential genes across different cancer types, which may help develop generic anticancer drugs that benefit a wide range of patients. Deduct the shared essential gene, CRISPR-Cas9 technology offers a chance to identify the context-specific fitness genes to specific tumor cells. As an example, one of the recent studies has compared four cancer types. The results indicate several context-specific fitness genes in glioblastoma, colorectal carcinoma, cervical carcinoma, and melanoma highlighting the potential for using genome sequencing and/or functional genomic screens to stratify patients (Guo et al., 2017). The increased expression of prosurvival

proteins (BCL-2, BCL-XL, MCL-1, or BFL-1/A1) is found associated with tumor escape apoptosis. The application of CRISPR-Cas9 technology not only evaluates the rationale of MCL-1 inhibitors (Merino et al., 2017). It also validates the combination of BFL-1/A1 and ataxia-telangiectasia-mutated ATM inhibitor as a synergistic method for disrupting apoptosis resistance in cancer (Guerra et al., 2018).

It also validates the combination of BFL-1/A1 and ataxia-telangiectasia-mutated ATM inhibitor as a synergistic method for disrupting apoptosis resistance in cancer(Guerra et al., 2018). TTK protein kinase (TTK) regulates the spindle assembly checkpoint (SAC) functions on maintaining genomic integrity. Inhibition of TTK in these tumors will cause synthetic lethality by abrogating the SAC and consequently increase aneuploidy to extreme levels that lead to cancer cell death. Using CRISPR screen to evaluate several TTK inhibitor (TTKis) followed by validation and correlative analyses in TNBC models aim to understand the mechanisms mediating TTKi sensitivity and resistance. The result indicates the APC/C as a central complex mediating sensitivity to CFI-402257 is directly relevant to the rationale for TTK inhibition in TNBC. The inherent genomic instability of these tumors was identified as a therapeutic vulnerability that can be exploited by TTK-targeting agents (Thu et al., 2018).

CRISPR-Cas9 also helped to correlate functional genomic data with known pathological features to help build the specific genetic mutations models for rare tumors (Guo et al., 2017).

1.5.2.1.2 Explore mechanisms of drug resistance

Drug resistance in tumour cells is associated with a wide range of cellular mechanisms include epithelial-mesenchymal transition (EMT), changes in autophagy and glycolysis, suppression of apoptosis, epigenetic modifications and alteration in the drug metabolism (Saber et al., 2019). When treating patients with drugs that target specific oncogenes,

those genomic changes triggered by treatment or the heterogeneity of the tumor itself can make cancer cells resistant to medications (Saber et al., 2019).

To overcome drug resistance, a clarification of drug resistance mechanisms is essential. Therefore, the CRISPR library applies for this type of screen to either evaluate newly synthetic drugs or test random mutations to investigate new possible therapeutic targets (Kurata et al., 2018). Genome-wide CRISPRa and CRISPRn screens were used to examine the impact of gain or loss of function in different genes to tumor drug response. by single nucleotide level genomic DNA modify or knocking out a specific gene, the CRISPR-Cas9 system provides an opportunity to study the mechanism of drug resistance in different cancer types. This utilization results in the identification of several resistance-related genes, which can result in desensitization when being overexpression, and are found to be able to be disrupted or be pharmaceutically inhibited genetically (Saber et al., 2019).

As an example, fibrous sheath interacting protein (FSIP1) is a cancer antigen expressed in the majority of breast cancer tissues and is associated with a poor prognosis. Studies use CRISPR-Cas9 to mediate the knockout of FSIP1 results in the observation of significant inhibition on the proliferation and invasion of triple-negative breast cancer (TNBC) cells and have on the chemotherapy-induced growth inhibition in vivo. FSIP1 deficiency promoted autophagy and enhanced AMP-activated protein kinase (AMPK) signaling, further by knockdown of AMPK or inhibition of autophagy restored the sensitivity to chemotherapy drugs in TNBC cells. Those results help to identify the role of FSIP1 in drug resistance also uncover its mechanism of influence the drug sensitivity (C. Liu et al., 2018).

The contribution of the DNA damage response (DDR) pathway to cellular platinum resistance was identified using a CRISPR screen. Some of the studies results indicate that in addition to homologous recombination, the Fanconi anemia and trans-injury

synthesis pathway, transcription-coupled nucleotide excision repair (TC-NER) and base excision repair (BER) contribute to platinum-based drug resistance. Those results may be considered as newly promising chemotherapy targets (Slyskova et al., 2018).

1.5.2.1.3 Examining noncoding regulators of cancer gene expression

apart from the protein-coding gene itself, many regions of the non-coding genome are involved in the regulation of protein-encoding gene expression. CRISPR-Cas9 can be used to identify the potential use of these non-coding regions in cancer therapy.

For example, The MYC and GATA1 oncogenes have been recognized to be associated with the progress of many different cancers. By using CRISPRi, nine distal enhancers have been identified within a one megabase sequence near the MYC and GATA1 oncogenes. Those noncoding regulator could be manipulated and further been employed for new cancer treatment strategies.

Some studies use CRISPRn to examine non-coding regulators that play a role in T cell failure to find a treatment method that prevents T cells from being inactivated by tumor cells in cancer immunotherapy. Through mutating all possible sgRNA sites, there encoding nine regulatory sequences near the Pdecd1 gene have been found which possibly used to suppressing the immune checkpoint pathway by upstream therapeutic intervention.

Another example is the use of CRISPR-Cas9 to knock out Nrf2 as for impairing the malignant phenotype of K-ras G12V transformed cells. Oncogene K-ras can cause malignant transformation when it is activated abnormally. Studies demonstrate, in cells carrying oncogenic K-ras, Nrf2 participates in the promoting cells survival and drug resistance. Based on this result, inhibition of Nrf2 may be an attractive strategy to increase the therapeutic effect and overcome the resistance of carcinogenic K-ras activation in cancer.

Except targeting enhancer bind sites, CRISPR screening with saturation mutagenesis or deletion can also detect a variety of other types of oncogenic modulators, including long noncoding RNAs (IncRNAs), microRNAs (miRNAs) and other critical Noncoding regions, such as introns and untranslated exons (Shao et al., 2018).

1.5.2.2 Application and advances of CRISPR-Cas9 in cancer immunotherapy

One of the most compelling uses of CRISPR-Cas9 technology in cancer therapy is its inclusion in cancer immunotherapy. Currently, cancer immunotherapy is one of the most promising cancer treatment strategies.

The immune system, particularly T lymphocytes, plays an essential role in identified tumor-specific antigens. In this case, the immune system of a patient can be used for eradicating cancerous cells (Mollanoori, Shahraki, Rahmati, & Teimourian, 2018). Time-consuming and expensive processes limit the utilization of autologous CAR-T cells. To overcome this limitation and simplify the manufacture of engineered CAR-T cells, the idea of making universal or of-the-self T cells. In other words, genetically modified allogeneic T cells from healthy donors replace the collection of high quality and quantity of T cells from patients (Mollanoori et al., 2018).

CRISPR-Cas9 is mainly applied for nullify immunosuppression in the tumor environment or to enhance the response of cytolytic lymphocytes to the tumor by disrupting multiple loci to generate universal CAR-T donor cells (Joshi & Durden, 2019).

Graft-versus-host disease (GVHD) reject infused allogeneic T cells in the recipient also trigger the improvement of biosafety profile for more great disease-targeted activity CAR-T donor cells. The endogenous αβ T-cell receptor (TCRs) on allogeneic T cell act to recognize recipient alloantigen (Mollanoori et al., 2018). Study shows using

CRISPR-Cas9 to knock out TCR-β, and β-2-microglobulin (B2M) result in the excluding of GVHD reaction (Ren, Liu, et al., 2017).

CRISPR-Cas9 can also be applied to eliminate the gene that encodes immune checkpoint molecules. For example, programmed cell death protein 1 (PD-1) and programmed cell death protein 1 (CTLA-4) both expressed in T cells and B cells. Those proteins can interact with its ligand (PDL-1), a transmembrane protein that expressed on cells membrane. Thus, to protect the cell from immune attack by promotes the apoptosis of antigen-specific T cells in lymph nodes and suppressive T cells (Loise M, Peter T, & Arlene H, 2010). The expression of checkpoint molecules on CAR-T cells leads to the exhaustion of T-cells. Besides, tumor cells could go up to regular the expression of PDL-1, thus result in the reduction of the immune response. Using CRISPR-Cas9 targeted nucleotide substitutions have been stained at the PD-1 locus of primary T cells, resulting in enhanced T cells effector function. The clinical trial using CRISPR-Cas9 mediated PD-1 knockout T-cells in lung cancer patients (Cyranoski, 2016). Ongoing clinical trials include PD-1 knockout autologous T cells in prostate cancer, bladder cancer, and renal cell carcinoma. The universal T-donor cells are successful generate by using CRISPR-Cas9simultaneous knockout of 4 loci of PD-1 and CTLA-4 (Ren, Zhang, et al., 2017). CRISPR-Cas9 mediated generation of lymphocyte-activating gene-3 (LAG-3) knockout CAR-T cell shows better specificity and antitumor potential in a xenograft mouse model (C. Zhang, Liu, Zhong, & Zhang, 2017).

Similar to immune checkpoint molecules, the Fas receptor (CD95) is a type of protein that able to induce T-cells apoptosis when it is binding with its ligand (FasL) (Mollanoori et al., 2018). CRISPR-Cas9 technology is introduced to overcome Fas/FasL-dependent activation-induced cell death (AICD) by producing Fas knock our CAR-T cells. These strategies improve tumor cell death and prolonged survival in mice. The Intrinsic human leukocyte antigen (Krall et al.) from allogeneic CAR-T cells can be

recognized as exogenous HLA and induces an immune reaction. This affection prevents its application as an autologous CAR-T cell in an allogeneic environment.

In conclusion, the CRISPR-Cas9 system, as a formidable genome editing tool, has been exploited to be a function-based large-scale screening strategy in mammalian cells(Baliou et al., 2018). CRISPR-Cas9 mediated generation of CAR into the T -cells receptor alpha constant (TRAC) locus showed a regular CAR expression in T-cell. The results show an increase in the efficiency of T-cells and can retent in an AML mouse model (Ghosh et al., 2019). This method has significant implications for personalized cancer therapy. There genetically modified T cells carrying tumor-targeting receptors have achieved positive therapeutic outcomes in patients with various hematological malignancies such as leukemia and lymphomas(Ghosh et al., 2019).

1.5.2.3 CRISPR-Cas9 technology with tumour stem cells

1.5.3 Challenges for completely therapeutic implementation

Although CRISPR-Cas9 technology has shown its potential in various cancer treatments, it still faces many difficulties before fully converting it into clinical applications, mainly related to its safety and efficacy of the therapy (Cox, Platt, & Zhang, 2015). Since the molecular nature of CRISPR-Cas 9 treatments is distinctly different from other small-molecules and biologic therapies, a synergetic engineering development in several areas will be needed for these tools be successfully brought to clinical used.

1.5.3.1 Factors influencing therapeutic efficacy

As previously mentioned, the CRISPR-Cas9 technology has shown promising advances in past clinical trials, but numerous factors can still affecting its effectiveness and safety for its entire clinical application. Those factors are mainly related to the fitness of the editing cell, editing efficiency, delivery methods, and potential off-target effects.

1.5.3.1.1 Fitness of edited cells

The fitness change of the edited cells is one of the difficulties for CRISPR-Cas9 clinical use. This fitness change means that genetically edited cells may become more or less adaptive to gene editing (Fig. 1.5).

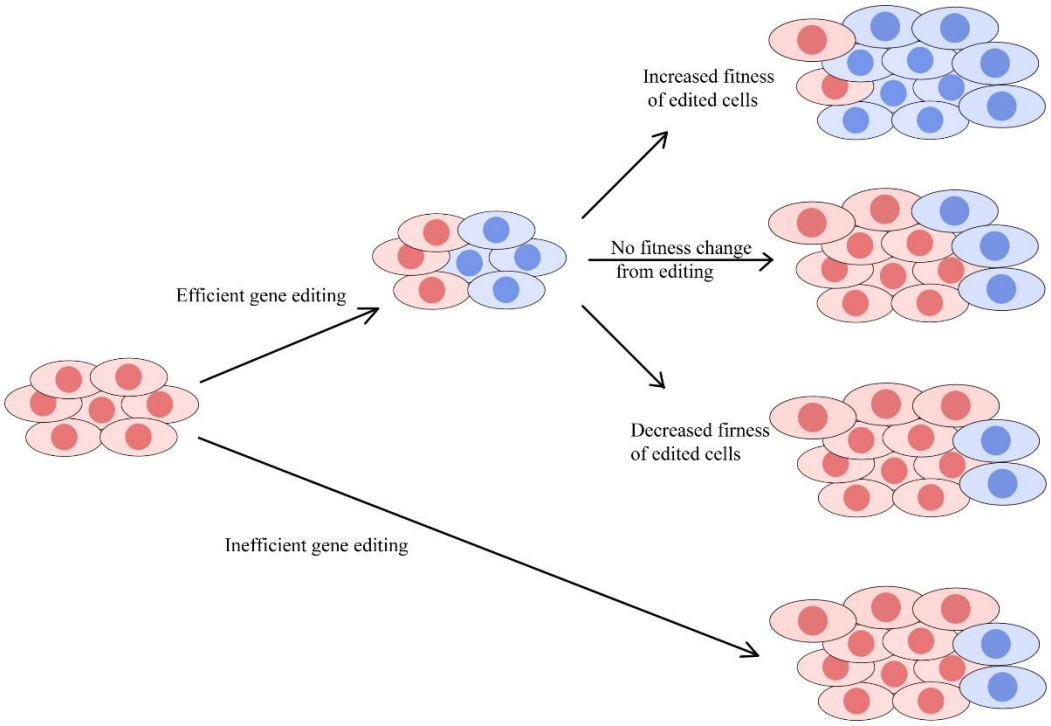

Figure 1. 6 The fitness of the edited influence the gene editing therapy efficacy.

If the edited cells have a better proliferation capacity, greater adaptability, and higher viability compare with unedited cells, they will gain a selective advantage (Cox et al., 2015). This positive fitness change reducing the total number of cells that initially need to be edited (Gonçalves et al., 2019), and further reduce the requirement for high editing efficiency (Xia et al., 2019).

In the opposite case, the edited cells turn to have fitness defects with weaker proliferation and differentiation capabilities compare with those unedited cells. These negative changes increase the correction of the mutated tumor suppressor genes and

causing the modified cells would be outcompeted by their diseased counterparts, thereby, result in a higher modification threshold and reduces the treatment efficiency. In all, this type of disease may not be suitable for gene editing therapy (Xia et al., 2019). In some types of cancer, the edited cells do not exhibit a change in their fitness, proliferation, or differentiation, which means the number of cells that initial need to be edited for therapeutic effect is slatily increased. In this case, although the treatment benefits can be reduced when the modified cell be defeated b their diseased counterparts, the disease is still hopeful to be cured by gene-editing therapy. However, especially in cancer cells, they have greater growth advantages than normal cells, including rapid proliferation. The instability can induce synthetic-lethal dependencies on genes that otherwise have no impact on cellular fitness. The changes of gene copy-number are rare in healthy cells but are one of the most common types of genomic alterations in cancer cells. For CRISPR-Cas9 technical based gene editing therapy, will require high editing efficiency. Therefore, this therapeutic threshold will be extremely high in that it demands modification of large numbers of the cell directly (M. Chen et al., 2019).

In general, given the current state of the technology, two situations are suitable for genome editing applications: either gene editing can confer a fitness advantage on cells, or small changes can influence clinical outcomes in the level of genetically edited products. This problem can be partially solved by in vitro CRISPR-mediated genome editing. When the edited cells are sufficiently expanded, they can be reinfused into the patient. However, spontaneous mutation of p53 may occur in the edited cells, and Cas9 may induce a p53-mediated DNA damage response. Therefore, it is critical to monitor the function of p53 to ensure that the patient's cells have functional p53 before and after engineering (M. Chen et al., 2019).

1.5.3.1.2 Editing efficiency

The activity of the DSBs repair pathway plays a significant role in controlling the editing rate. As motioned, NHEJ, and HDR are the two primary DSBs repair pathways. The NHEJ repair pathway induces out-of-frame mutations or premature stop codons and is, therefore, suitable for gene knockout studies (Gonçalves et al., 2019). In contrast, HDR mediated repair pathway is ideal for targeting transgenes, replacing specific genomic mutations with specific exogenous (Devkota, 2018).

The chooses of NHEJ or HDR-mediated repair mechanisms will cause a noticeable difference in editing efficiency. This effect can exhibit not only in different cell types but also in different cell states. Compare to the HDR pathway, NHEJ pathways are more active and efficiency in most cell types.

In germ cells following DSB, replication will occur on homologous chromosomes, which increases the chance of binding homologous chromosomes into the genome. In somatic cells, it is always preferred for the replication from the same sister chromatids. In this case, the integration rate of donor DNA is low. On the other hand, since sister chromatids can be used as S/G2, HDR is limited to these stages of the cell cycle. The use of sequences from sister chromatids or homologous chromosomes can improve the accuracy of DNA repair in the HDR repair pathway (Devkota, 2018).

In short, NHEJ is generally more flexible and efficient for generating indels to knockout carcinogenic gene than HDR. As an accurate genetic modification, HDR is relatively slow. The activity of the DSB repair pathway determines the speed of gene editing, which affects the efficacy of most gene-editing processes. As mentioned previously, the NHEJ-mediated channel is more active and efficient in most cell types than the HDR-mediated repair pathway. In this case, one of the main challenges is to improve the efficiency of the HDR repair pathways (Cox et al., 2015).

The DNA repair machinery can be modified to either favor NHEJ or HDR according to the requirement of the experimental design. Multiple strategies have applied for the increase of HDR repair pathway, include either activate the HDR using chemically and genetically or suppress the activity of NHEJ.

Cells stop the at S/G2 cell cycle stage to provide more time for HDR. By attaching degron to Cas9 for degradation at all stages of the cell cycle where HDR is absent, the cas9 induced DSB only happened at this stage. By using the selection marker to enrich the correctly edited cells and also increasing the concentration of donor DNA near the cut sites to increase the probability of the utilization of donor DNA as a repair template (Cox et al., 2015).

1.5.3.1.3 Delivery methods, especially in vivo delivery

For clinical applications, the selection of appropriate delivery methods is another challenge for the comprehensive implementation of CRISPR-Cas9 mediated gene editing therapy. Current, three basic delivery strategies are mainly being reported. Include the delivery of DNA plasmid encoding both the Cas9 protein and guide RNA, the delivery of mRNA for Cas9 translation alongside a separate guide RNA and (Levine, Rieger, & McCluskey) the delivery of Cas9 protein with guide RNA (ribonucleoprotein complex). Suitable delivery methods are selected based on the type of compound desired to be delivered and in vitro and/or in vivo use (Christopher, Jason, James, & Jerilyn, 2018). The success of gene editing is attributed to the effectiveness of cell targeting, rapid clearance, and minimal cytotoxicity after working in the CRISPR system. However, it is difficult to achieve all of the above criteria simultaneously using current delivery methods (M. Chen et al., 2019). The occur of off-target effects, and immune responses are needed to be reduced to achieve a sufficient clinical benefit. Also, the programmable nuclease needs to be delivered transiently in an appropriate

amount. Proper delivery methods can avoid possible off-target cleavage and immune response activity (Cox et al., 2015). Besides, the choice of delivery method affects the transient or permanent expression of the nuclease in the target cell.

There are generally three categories of delivery methods, including physical delivery, viral vectors, and non-viral vectors. The most common physical delivery methods are microinjection and electroporation. Viral delivery vectors include specifically engineered adeno-associated virus (AAV), and full-sized adenovirus and lentivirus. The viral vector was found to be the most common CRISPR-Cas9 delivery vectors for in vivo work. Non-viral vector delivery is not as prominent as viral-based delivery, but it possesses several advantages over viral vectors and is a burgeoning area of research. Non-viral vector systems include systems such as lipid nanoparticles, cell-penetrating peptides (CPPs), DNA 'nanoclews', and gold nanoparticles.

Physical delivery:

Microinjection is a high-efficiency delivery method (efficiencies approaching 100%). It can directly inject either plasmid DNA encoding Cas9 protein and the sgRNA, mRNA encoding Cas9 and sgRNA or Cas9 protein with sgRNA into individual cells. Also, this method is not to be limited by the molecular weight of the delivered complex, which is a significant limiting factor with viral vector delivery systems. This method also allows for the controlled delivery of known quantities of the delivered packages, which improving the control over off-target effects. This method is suitable for in vitro and ex vivo and applied for individual cells (Christopher et al., 2018).

Electroporation utilizes pulsed high-voltage electrical currents to transiently open nanometer-sized pores within the cellular membrane of cells suspended in a buffer — this method allowing for components with hydrodynamic diameters of tens of nanometers to flow into the cell. Electroporation is less dependent on cell type than other delivery techniques and can efficiently transfer cargo into cells that are

traditionally difficult to manipulate. Electroporation is most commonly used in an in vitro setting, though as with microinjection, ex vivo applications are also valid. Owing to the oftentimes-large amounts of voltage needed to be applied across cell membranes; however, electroporation is typically not suitable for in vivo applications (Christopher et al., 2018).

Non-viral vector:

Compare to plasmid DNA or viral vectors, Non-viral delivery has some potential advantages. It allows precise control of drug delivery duration, reduced long-term expression of nucleases, reduced the risk effects of off-target, and minimize the possibility of side effects. All of these support fast, safe, and efficient gene editing in the body. However, nanoparticle-mediated protein delivery is even more challenging than nucleic acid delivery. The process for packaging into small particles, maintaining biological activity, and then preventing their degradation before entering the nucleus will be more complicated. Therefore, and non-viral delivery material should be well-tolerated, biocompatible and non-immunogenic, and capable of delivering efficient Cas9-sgRNA to the core for genome editing.

Lipid nanoparticles have long been used for delivering a wide range of different molecules to cells and have demonstrated popularity for nucleic acid delivery.

Due to the characteristic of highly anionic nature, it is difficult for nucleic acids to pass through a cell membrane. However, by encapsulating nucleic acids within typically vary cationic, it would become reasonable easy for the delivery. Using non-viral delivery also minimize safety and immunogenicity concerns since the lipid nanoparticles do not contain any viral components. Like viral particles, non-viral allowing for extensive testing on a variety of scales of cell populations.

Despite the benefits, there are still substantial drawbacks for the delivery of CRISPR-Cas9 components via lipid nanoparticle. First, once the nanoparticle has passed through

the membrane of the target cell, it is typically encased within an endosome. The encased contents can quickly be directed into the lysosomal pathway by cell, which then causes lysosome content degradation. Second, if the Cas9-sgRNA complex can escape the endosome, it must also translocate to the nucleus, which can also be a potential point of failure. In this case, it is hard to achieve high efficacies when delivery CRISPR-Cas9 components via lipid nanoparticles. Finally, lipid nanoparticles are like virus particles. Both the size of the cargo and the target cell type would profoundly affect the efficiency of transfection and the selection of appropriate lipids types for the use in the system (Christopher et al., 2018).

Viral vector:

Viral vectors, such as an adeno-associated virus (AAV), is one of the most widely applied vectors in current studies. AAV achieves highly efficient in vivo delivery of CRISPR-Cas9 and has recently been approved for clinical use. AAVs come in many serotypes and able to deliver a variety of tissue types including the sys, brain, liver, and muscle. For Cas9, short orthologs may be packaged along with guide RNAs into a single AAV. The advantages of AAV include low immunogenicity, low risk of carcinogenesis, and serotype-associated target cell specificity (Cox et al., 2015). Evidence has proved that each AAV serotype has its preferential delivery efficiency for a particular cell type. thus, researchers can choose the optimal serotype to enhance targeted delivery to the organ of interest

However, the application of AAV still faces difficulty. First, AAV-mediated nuclease expression is often constitutive, whereas it would be desirable to shut down nuclease expression after a successful genome editing. Second, those patients who have already been exposed to AAV may develop immunity against specific serotypes

and no longer suitable for AAV mediate delivery. Using viral-mediated Cas9 delivery may result in constitutive expression of nuclease proteins and further cause genome

instability and toxicity. self-cleaving mechanisms may be used to inactivate the nuclease transgene on the delivery vector (Cox et al., 2015).

Although the well performs of AAV-mediated Cas9 delivery in a laboratory setting, there are still some concerns with the use of AAV in the clinical trials. First, it may inadvertently disrupt the expression of an essential gene when the transgene is integrated into the target genome. Second, the maximum capacity of AAV is limited. Therefore, it is challenging to construct a virus that can encode such a large gene, its promoter, and gRNA. By delivery purified Cas9-sgRNA ribonucleoprotein (RNP), a long-term presence of viral-temporal control of CRISPR activity can be achieved. Since RNP can rapidly degrade in a few hours, it ensures a higher editing efficiency compare with DNA or RNA based distribution.

1.5.3.1.4 Off-target effects

Off-target effects (OTEs) can be triggered by multiple events, which include the DNA cleavage or binging at unintended genomic sites, and subsequent editing or regulatory events. Previous studies indicate that OTEs can frequently generate indels at undesired genomic loci.

The specificity of the CRISPR-related nuclease depends on a variety of factors, including the genomic locus, host cell type, culture conditions, and the dose and duration of nuclease present. The continued genetic modification increases the risk of off-target cleavage and reduces editorial specificity, which may result in unnecessary mutations and potential toxicity. This effect may even worsen when combined with other sources of experimental variation, including clonal variations in the cellular system and assays used. Furthermore, CRISPR-Cas9 showed a higher risk of off-target effects in human cells compared to the other two methods used for genome editing, zinc finger nucleases (ZFNs) and TALENs. Off-target effects are associated with genomic

toxicity, carcinogenesis, genomic instability, functional gene disruptions, epigenetic changes, cell death, and cell transformation (Karimian et al., 2019).

The success of gene editing can be judged by targeted analysis of genomic loci and detection of off-target effects. Although those techniques used for assessing the specificity of particular gRNA, the off-target effects will still impact the interpretation of the experimental results. In this case, it is more crucial to reduce and control off-target effects. Previous studies have worked out strategies by applying a range of design tools, engineered reagents, as well as experimental procedures to overcome the off-target effects.

The successful on-target changes in genome-edited cells can be readily monitored by targeted analysis of the genomic loci by several assays. Such OTE detection and mapping efforts allow assessment of the specificity of a particular gRNA. However, it is still unclear to what extent the detected OTEs will impact the interpretation of the experimental results. More practically, CRISPR OTEs can be reduced and controlled by using a range of design tools, engineered reagents, as well as experimental procedures. To minimize the off-target effect, either can increase the specificity of nuclease-mediated cleavage of the target site or restrict the duration of nuclease expression to reduce the possibility of off-target mutations (Xia et al., 2019).

Multiple strategies are applied for the experimental design and down-stream phenotypic assessment to reduce the OTEs and derive meaningful experimental conclusions. One of the first essential steps for reducing OTEs in genome editing is a proper selection of the guide RNA (gRNA) utilized to direct the Cas9 nuclease to the target gene. The target sequences should be select with lower homology, and the dosage of CRISPR-Cas9 should be carefully controlled. The structure and composition of gRNA can affect the level of off-target effects, using truncated sgRNAs at 5' and by two to three nucleotides that are sensitive to mismatches reduces off-target effects. Based on early

reports, the tolerant of cas9 for mismatches between sg RNA and target sequence depending on mismatches position and the identity of their nucleotides. Because CRISPR-Cas9 applies changes are permanent, off-target effects must be carefully controlled (Karimian et al., 2019).

The application of high-fidelity Cas9 nuclease variants showed a pronounced reduction in off-target cleavage events while retaining comparable on-target editing capability to the original version of Cas9. The choose of the delivery method associated with the activities of nuclease in host cells results in different levels and duration of express for both Cas9 and gRNA. Cell lines that can stably and constitutively expressing Cas9 used for gene knock out. The sustained and robust expression of Cas9 increases the efficiency of gene editing. However, the off-target effect is also increased due to the continuous and high-level expression of Cas9 nucleases that promote gRNA/target mismatch tolerance (Kimberland et al., 2018).

Although measures can be taken to reduce OTE, the prior art cannot be avoided entirely. For this reason, it is recommended to take steps in the experimental design to control these effects to increase confidence in the experimental results. However, information from such controls is limited to determining the extent of OTE caused by Cas9 or its expression, rather than controlling OTE from a particular gRNA used. Besides, random or out-of-order gRNA may introduce unexpected insertions that may result in unnecessary phenotypic results.

In summary, as a powerful gene-editing tool, CRISPR-Cas9 has shown its potential in various anticancer therapies. Despite some challenges, we can still believe that by systematically optimizing its efficacy, safety and specificity can lead to the development of biological treatments and improve the patient's future treatment.

1.6 Aims

Currently, it is not possible to predict which patients will respond poorly to oxaliplatin. We hypothesise that MRP2 extrudes oxaliplatin from tumour cells thereby limiting its cellular accumulation and cytotoxicity. This thesis investigates 1) whether CRISPR-Cas9 technology can be applied for knocking-out MRP2 in HepG2 cell line and 2) whether knocking out MRP2 can abolish its function and may improve the efficacy of oxaliplatin in HepG2 cell line.

Chapter 2 Materials and methods

2.1 Chemicals and reagents

Chemicals including chemical compounds, reagents, buffers, and solutions used in this study together with their sources and suppliers were as listed below in table 2.1

Table 2. 1 Chemicals used in this study with their sources

Chemicals	Sources(suppliers)
Ethanol	
Lipofectamine ™ CRISPRMAX™ Cas9 transfection reagent (Cas9 Plus™ lipofectamine and CRISPRMAX™ lipofectamine)	ThermoFisher (Pub.No.MAN0014545)
TrueCut™ Cas9 Protein v2	ThermoFisher (Pub.No.MAN0017066)
RPMI	ThermoFisher
Opti-MEM medium	ThermoFisher
Fetal Bovine Serum (FBS)	In vitro Technologies, Auckland, NZ
TrypLE™ express enzyme	ThermoFisher
GeneArt® genomic cleavage detection kit (GCD)	ThermoFisher

Table 2. 2 Two ABCC2 CRISPR/Cas9 sgRNA sequences and the corresponding genomic cleavage detection primer oligos.

Added In Cart	Gene	CRISPR Sequence	Direction	PAM	Binding Sites	gRNA Forma
	ABCC2	GGATTGGTATATCGAACAGC	(-)	AGG	2	GeneArt™ P.

	GCD Primer	Forward Oligo	Reverse Oligo	GCD Band Sizes
✔	Set1	CAGAAGTACATCCCTAAGTC	TGTTTCACCCCATTCCAA	600 [406 bp,194 bp]

Added In Cart	Gene	CRISPR Sequence	Direction	PAM	Binding Sites	gRNA Format
	ABCC2	TGGTCCTGGATTTATACACG	(-)	TGG	3	GeneArt™ P...

	GCD Primer	Forward Oligo	Reverse Oligo	GCD Band Sizes
✔	Set1	AATGCAGTGGTGAGGTTTAC	AGAAATGCAGCAAGGGAC	628 [405 bp,223 bp]

2.2 Cell lines and cell culture

Human liver carcinoma HepG2 cells were grown in complete Roswell Park Memorial Institute (RPMI) medium which supplemented with 100 units/ml penicillin G sodium, 100 µg/ml streptomycin, 4 mM L-glutamine, and 10% fetal bovine serum (FBS) at 37℃ in a humidified atmosphere with 5% of CO_2.

2.3 Transfection

To establish a knockout cell line, the lipofectamine-based delivery of TrueCut™ Cas9 Protein and TrueGuide™ sgRNA (3 nmol) were applied according to the manufacturer' instructions. The development of containment GMOs is approved by NZ Environment Protection Authority with the approval number GMD102074.

2.3.1 Preparation of working stock of sgRNA

To rehydrated the dried sgRNA (3nmol), 30µL of nuclease-free 1 × TE buffer was added in each sgRNA tube to make a final concentration of 100µM (100pmol/µL). For the transfection purpose, the sgRNA stock solution was diluted to a working solution using nuclease-free water in a sterile microcentrifuge tube. 6 µL of 100µM sgRNA stock solution was added in 14µL of nuclease-free water to make 20µL of 30

μM sgRNA (30pmol/ μL) working concentration. The diluted sgRNA working solution was freshly prepared andstored at -20℃ for no more than 3 months.

2.3.2 Seeding cell

Once the cells reached 70-80% confluence, the culture medium was aspirated off and the cells were washed with pre-warmed sterile PBS (phosphate buffered saline). After washing, 2.5 ml trypsin was added to cover the cells at the bottom of the flask and the flask was placed in an incubator at 37 ℃ for 5-10 minutes. After the cells were detached, the reaction was stopped with 2.5 ml of ice-cold culture medium and all amounts of the suspension were transferred to a 15 ml centrifuge tube for centrifugation at 200 *g* for about 5 min. The supernatant was removed and 1 ml of complete Opti-MEM I medium without antibiotics was added to the tube to resuspend the cells. 10 μL of the suspension was taken out using a pipette, placed on a piece of Parafilm and thoroughly mixed with 10 μL of Trypan Blue. 10 μL of this mixture was added to one side of the hemocytometer until the chamber below the coverslip was filled.

Take the average number of cells in each region, then multiply by 10,000 and multiply by 2 to correct the 1:2 dilution of Trypan Blue addition. Calculate the total number of cells in 1 ml of the medium by the following equation:

Number of cells/ml = average number of cells per square × 2 × 10^4

HepG2 cells were seeded at a density of 1.2 × 10^5 cells/well in five wells in a 12-well plate.

2.3.3 Transfection

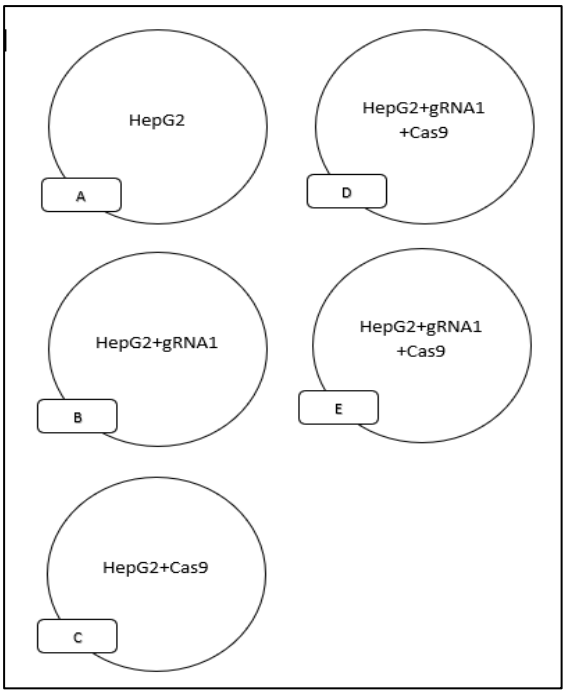

Figure 2. 1 Layout of a typical transfection experiment using a 6-well plate.

Five microcentrifuge tubes were prepared as following:

The control group (A): 2.5 µL of Cas9 Plus lipofectamine was added to 50 µLOpti-MEM medium, followed by 25 µL of Opti-MEM containing 2.5 µL of CRISPRMAX lipofectamine. The mixture was incubated for 15 minutes at room temperature and added to the cells.

The control group (B): 1 µL of working stock gRNA and 2.5 µL of Cas9Plus lipofectamine were added to 50 µL of Opti-MEM, followed by addition of Opti-MEM containing 2.5 µL of CRISPRMAX lipofectamine. The mixture was incubated for 15 minutes at room temperature and added to the cells.

The control group (C): 1250 µL of Cas9 and 2.5 µL of Cas9Plus lipofectamine were added to 50 µL of Opti-MEM, followed by 25 µL of Opti-MEM containing 2.5 µL of CRISPRMAX lipofectamine. The mixture was incubated for 15 minutes at room temperature and then added to the cells.

The ABCC2-KO group (D & E): 1250 ng of Cas9 and 2.5 μL of Cas9Plus lipofectamine were added to 50μL Opti-MEM medium. The molar ratio of gRNA to Cas9 protein is maintained at about 1 to 1.2:1. The tube was gently tapped several times to mix the samples and then incubated for 10 minutes at room temperature. A pre-prepared mixture of 2.5 μL of LCRISARTMAX lipofectamine with 50 μL of Opti-MEM medium was added to each tube, and then incubated at room temperature for 15 minutes, and finally added to the cells.

All solutions were mixed by gently swirling the plates, and then the plates were incubated at 37 ° C for 48 hours in a 5% CO_2 incubator. After the incubation, the medium was removed, and the cells were washed with 500 μL of sterile pre-warmed PBS.

2.4 Fluorescent probe-based assay for validation of MRP2 function

This study will assess the activity of the MRP2 transporter by measuring CDCF accumulation in in MRP2-KO and control HepG2 cells.

When the cells in the flask grew to about 80-90% confluence, they were washed twice with PBS and were trypsinized. After the cells detached, they were transferred to a 15 mL centrifuge tube and centrifuged at 200 *g* for 5 min. The cells were then washed once with PBS and resuspended in 1 mL of Opti-MEM medium. The cells were being counted and transfer in a centrifuge for the final cells number at around 10^6 cells. Accumulation of CDCF was initiated by mixing the cells with 3 μL of 1 mM CDCFDA (0.1% DMSO) for 20 minutes at 37 °C. The accumulation was terminated by adding 3 mL of ice-cold PBS and centrifuging at 500 *g* for 5 min. The cells were again washed with 3 mL of ice-cold PBS, then the cells were resuspended in 0.4 mL of ice-cold PBS and immediately placed in ice. The intracellular level of CDCF was analyzed using the MoFlo XDP flow cytometer (Beckman Courter) equipped with a standard argon laser

for excitation at 488nm, and a bandpass filter at 525nm was used to detect CDCFfluorescence.

To gate out cell debris and doublets, multiple gating of cells was performed. Cells of a defined range of sizes and only single cells were detected in each flow cytometric analysis. Single cells were selected by gating on the events along the diagonal in the signal pulse height versus area plots for forward scatter (FSC) and side scatter (SSC) (Figure 2.2). An example of data acquisition and definitions of gated populations are shown in Figure 2.4. Fluorescence intensity was expressed as the geometric mean value of 10,000 events from gated population C.

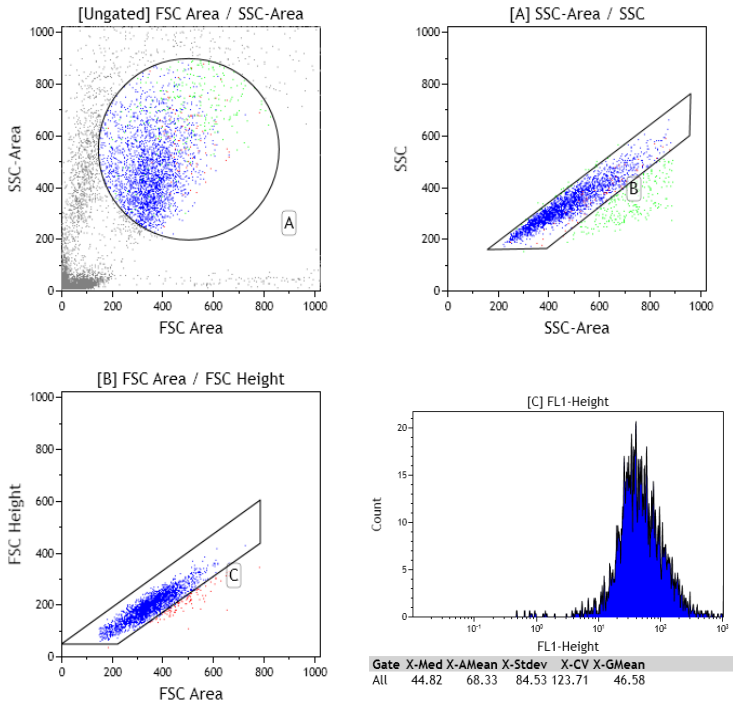

Figure 2. 2 An example of flow cytometric data acquisition and analysis using the software Kaluza. A) A dot plot of analysed cells based on signal pulse area of the forward scatter (FSC) and side scatter (SSC). A population of cells were selected so that only cells that were in the gating were analysed. These cells were designated as population A. B) Population B was gated as the signal pulse height versus area of the SSC. Single cells, which were located along the diagonal of the plot, were designated as population B.C) the same procedure as in in B) was repeated for FSC, and the selected cells were marked as population C. D) Population C was used in the analysis of cellular fluorescence intensity.

2.5 Verification of gene editing efficiency

In this study, we used the gene art genomic cleavage detection kit that purchased from Thermo Fisher as the tool to verify the gene editing efficiency. this kit was applied according to the manufacturer instructions.

The basic concept of this assay is to uses the genomic DNA that extracted from cells transfected with ABCC2 guide-RNA/CAS9 protein ribonucleoprotein complexes through liposome-mediated delivery. Those cells treated with CRISPR/Cas9were following with cleavage, genomic insertion or deletion (indels) that created by the cellular repair mechanisms. Loci where the gene specific DSB happened are amplified by PCR. After the denaturing and reannealing of the PCR product, the mismatches were generated as strands with an indel re-annealed to strands with no indel or a different indel. The mismatches were subsequently detected and cleaved by the detection enzyme and then the resultant bands subsequently analysed by gel electrophoresis and band densitometry. The gene editing rate is then calculated by using band densitometries.

2.5.1 Harvest cells

The cells that ready for detection was first being spun down at 200 × *g* for 5 min at 4 ℃. Then the supernatant is carefully removed and proceed to lysis or store pellet at -80°C prepare for the use.

The genomic DNA at the locus is first being PCR amplified prior to detection, and to achieve the optimal amplification and subsequent detection, the assay is follow the recommended guideline.

The primers are 18–22 bp in length and have 45–60 % GC content for the best design with Tm >55° C. Also the design primers should be design to make the potential cleavage site not in the centre of the amplicon and the detection reaction will yield two distinct product bands.

2.5.2 Cell lysis and DNA extraction

For cell lysis, 10^6 cells were used. 50 µL of Cell Lysis Buffer with 2 µL of Protein Degrader were mixed in a microcentrifuge tube. Then 50 µL of the mixture was added to each cell pellet and resuspend it and transfer all of the resuspended cell pellet to a PCR tube. The PCR tube was then heated using the following program in a thermal cycler:

Temp	Time
68°C	15 min
95°C	10 min
4°C	Hold*

Following the completion, the PCR amplification was immediately proceeded.

2.5.3 PCR amplification

For the PCR amplification, first the cell lysate was briefly vortexed and then transferred to each PCR tube with the following other components:

Component	Sample	control
Cell lysate	2µL	_
10 µL F/R primer mix	1 µL	_
Control template & primers	_	1 µL
ampliTaq Gold 360 Master Mix	25 µL	25 µL
Water	22 µL	24 µL
Total	50 µL	50 µL

The PCR reaction was run with the following conditions:

Stage	Temp	Time	Cycles
Enzyme activation	95℃	10 min	1×
Denature	95℃	30 sec	40×
Anneal	55℃ (Tm)	30 sec	
Extend	72	30 sec	
Final extension	72	7 min	1×
Hold	4	Hold*	1×

2.5.4 Gel analysis

Prepare a 100 ml of agarose gel solution (concentration 2%) in a 250nl flack and mix it thoroughly. The flask was heated in the microwave until the solution is obvious and on small floating particles are visible (about 2~3 minutes). . Then 5μL of RedSafe™ Nucleic Acid Staining Solution (20,000×) was added to the agarose solution followed byswirling the flask gently to mix the solution and avoid forming bubbles. While the agarose solution cools, pour it into the gel tray until the comb teether is immersed about ¼~1/2 into the agarose. The thickness of the gel should be less than 0.5 cm since thick gels may decrease sensitivity. Then allowing the agarose gel to cooled until solidified. PCR samples were loaded on the gel and electrophoresis performed. Visualization of the DNA bands was under UV illumination

2.5.5 Verifying the PCR product

3 μL of PCR product with 10 μL water was applied on a 2% agarose gel. If a single band of the correct size is present, with an intensity similar to 50ng of 400bp band in the mass ladder, the denaturing and re-annealing step was undertaken, or the PCR product was stored at -20℃ for later use.

2.5.6 Cleavage assay

To randomly anneal the PCR fragments with and without indels to form heterogeneous DNA duplexes, 2 μL of PCR product was added with 1 μL 10X Detection Reaction Buffer and 6 μL water in a PCR tube. After brief centrifugation in a microfuge to ensure that no bubbles are present, the PCR tube was placed in a thermal cycler with a heated-lid and run the following program:

Stage	Temp	Time	Temp/time
1	95°C	5min	_
2	95°C-85°C	_	-2°C/sec
3	85°C-25°C	_	-0.1°C/sec
4	4°C	_	Hold*

Following completion of re-annealing reaction, enzyme digestion was initiated by mixing with 1 μL Detection Enzyme each to all test samples. 1 μL Water was added to all negative control samples. All samples were incubated at 37°C for 1 hour before vortexing briefly and spinning down. The entire 10 μL sample was immediately loaded on a 2% gel with 10 μL Water. Electrophoresis was run with a sizing standard 2 kb DNA ladder in parallel.

2.6 Methods for cell viability MTT Assay

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was used as the primary assay to measure viable cells in the experiments. In this assay, the tetrazolium salt is reduced by dehydrogenase enzymes in the mitochondria of living cells to produce purple formazan crystals, which were then dissolved in dimethyl sulfoxide (DMSO) solution. The absorbance or optical density (OD) of the solution was measured using a spectrophotometer at 540 nm and 680nm (reference) wavelength.

Preliminary experiments were performed to define the linear range between OD values and the number of viable cells.

To evaluate the cellular sensitivity to oxaliplatin-induced cytotoxicity,different cell lines (knockout cell line and wild type cell line) were exposed to oxaliplatin to varying concentrations for 2h followed by measurement of viable cells with MTT assay.

In this study, cells were seeded at 8,000 cells/well in a collagen-coated 96-well plate and allowed to attach to the well surface over 24h incubation in the drug-free growth medium. After attachment, cells were exposed to oxaliplatin at different concentrations and incubated for 2h at 37ºC with 5% CO_2. The medium was replaced with the drug-free growth medium to terminate drug exposure. Cells were then allowed to grow for 72 h to reach the optimal density, and then MTT assay was performed to determine cell viability.

The details of this assay are described in the following sections.

2.6.1 Seeding cells

Cells from both the experimental and control groups will be respectively plated in 96-wells plates for the MTT assay.

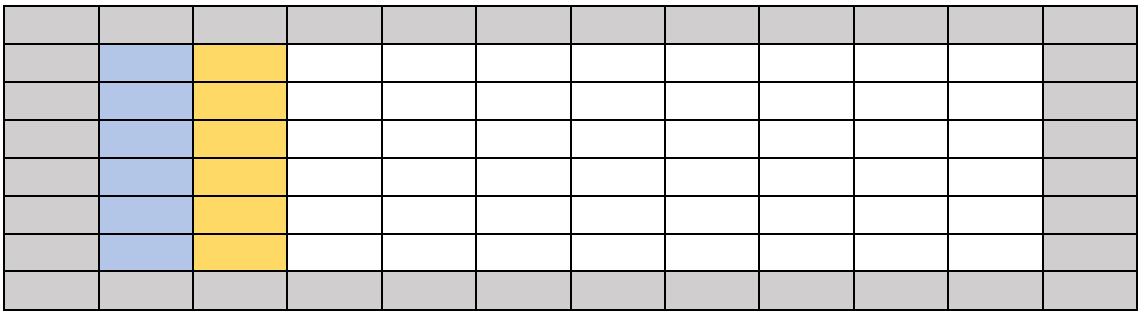

Table 2.1 Illustration of cell seeding in a 96-well plate. As shown in the table, the grey wells was loaded with 300 µl of sterile PBS each. The blue ones were used as the blank group which filling by the completed medium and the others was seeded with a density of 8,000 HepG2 cells per well, with the yellow ones serving as a drug-free control.

The plates were kept in an incubator, and the cells were incubated for 16-24 hours in normal drug-free growth medium to adhere to the surface of the plates.

2.6.2 Oxaliplatin treatmentment

After the attachment, cells were exposed to oxaliplatin at a designated concentration as 3.125μM, 6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM, 200 μM, and 400 μM respectively. The plates were put back to the incubator and kept for two hours at 37 ℃. The medium containing drug was then being removed and replaced by the drug-free growth medium to terminate drug exposure. Cells were then allowed to grow to reach the optimal density, which was at 72 h after the initial introduction of cells to the drug. The MTT assay was then performed to determine cell viability.

2.6.3 MTT Assay

Preparing 12mM of MTT stock solution by adding 1mL of PBS to 5mg vial of MTT. The complex was then mixed by vortexing or sonication until the MTT fully dissolved. Occasionally there may be some particulate material that will not be removed by filtration or centrifugation. MTT stock solution can be stored for a maximum of four weeks at 4℃, and each 5mg vial of MTT provides sufficient reagent for 100 texts. After 72 h of incubation, remove the entire medium from each well and replace with 100 μL of fresh phenol-red free culture medium. Adding 10 μL of 12mM MTT stock solution to each well include the negative control. After 4h of incubation, the cells were labeled with MTT as described above. Remove all but 25 μL of the medium from the wells and adding 150 μL of DMSO to each well and mix thoroughly using an orbit plate shaker. The plates are then incubating for additionally 10min, after the incubation, remix each sample and read absorbance at 540nm and 680nm (reference wavelength)

2.6.4 IC_{50} calculation method

The percent cell viability at different concentrations was calculated using the OD values measured from MTT assays, and the average OD value of untreated control cells was normalized to 100%. The percent viability values were used to plot the nonlinear dose-response (inhibition) curve, and the IC_{50} values (50% reduction in the cell viability

compared with untreated controlled cells) were determined from the curve fit using GraphPad Prism version 6.

In this study, the IC_{50} was calculated by the following equation:

$$Y = Min + (Max-Min)/(1+10^{((LogIC_{50}-X)\times H)})$$

where X stands for logarithm concentration; and H for Hill slope.

2.7 Statistical analysis

The data were presented as mean value with standard deviation (mean ± S.D). Linear and non-linear regression analyses were applied as appropriate using Prism 6 software (GraphPad, San Diego, CA, USA). To test whether the mean values were statistically significant or not, unpaired Student's t-test, one way or two way ANOVA with Tukey's multiple comparison post-tests were applied as appropriate. *P*-value of <0.05 was considered to indicate the results were statistically significant.

Chapter 3 Results

Our strategy is to investigate the feasibility of specifically knocking out ABCC2 alleles in the HepG2 cell line using CRISPR/cas9-based gene editing. Such cell lines can be generated by using a liposome-delivered CRISPR-Cas9 system, which allows for the precise and efficient creation of gene knock-outs with minimum off-target effects. It is essential to determine the genomic cleavage efficiency and functional changes in MRP2 knock-out cells line. Thus, in this study, we tried to eliminate ABCC2 genes by transfecting two different gRNAs individually with Cas9 and then assess the genomic cleavage efficiency. CDCF accumulation and MTT assay was used to compare the phenotypic changes between transfected and wild-type HepG2 cell lines.

3.1 Effect of *ABCC2* gRNA1/Cas9 transfection

3.1.1 CDCF accumulation as a surrogate for MRP2 function

To detect changes in MRP2 activity in cells after CRISPR-mediated gene editing. The knockout cell group and the wild type cell control group were exposed to a 3 μM CDCFDA for 20 minutes, respectively, and the accumulation of CDCF in the cells was measured using flow cytometer. The detailed steps are described in section 2.4.

As shown in Figure 3.1, the accumulation of CDCF in gRNA1/Cas9 transfected HepG2 cells was significantly lower than that in wild-type cells.

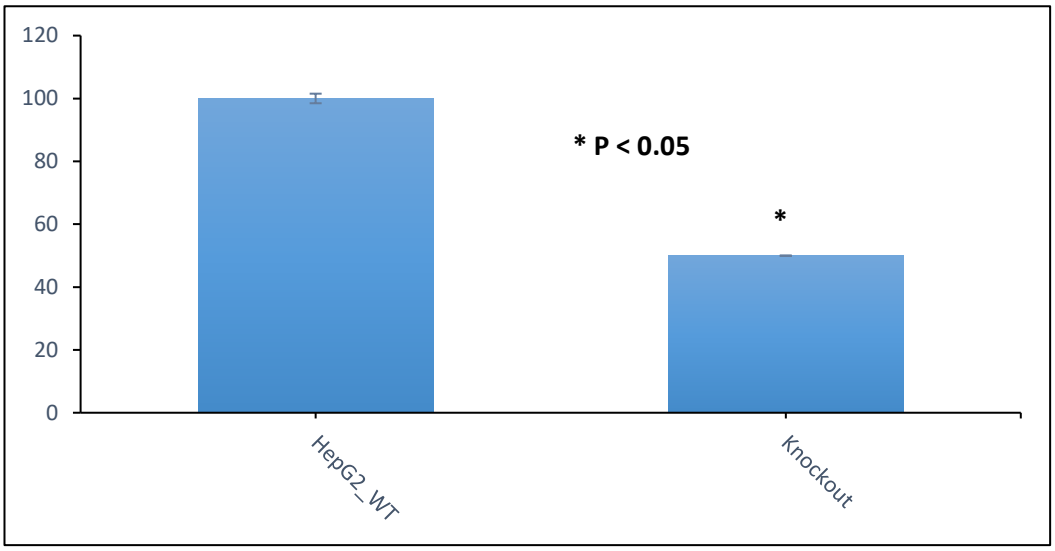

Figure 3. 1 Accumulation of CDCF, a model MRP2 substrate in wild-type and ABCC2 gRNA1-Cas9 transfected HepG2 cells. Data are means ± SD, n =3. * *P* < 0.05, student unpaired t-test

Under the same conditions, the CDCF accumulation in the "knockout" (gRNA1/Cas9 transfected) HepG2 cell line was lower than that of the wild-type cell line (HepG2_WT). This result was completely opposite to our hypothesis.

3.1.2 Effect of ABCC2 gene knockout on oxaliplatin cytotoxicity

In this study, MTT assay is used to analyse the potential changes in chemosensitivity to oxaliplatin in HepG2 transfected with ABCC2 gRNA1/Cas9. Wild type and MRP2 knockout cell lines were exposed to the increasing concentrations of oxaliplatin for 2h, and allow to grow for 72h in blank complete medium. After treatment, the number of cells was determined by measuring the absorbance. The results of the MTT assay are shown in the figures below.

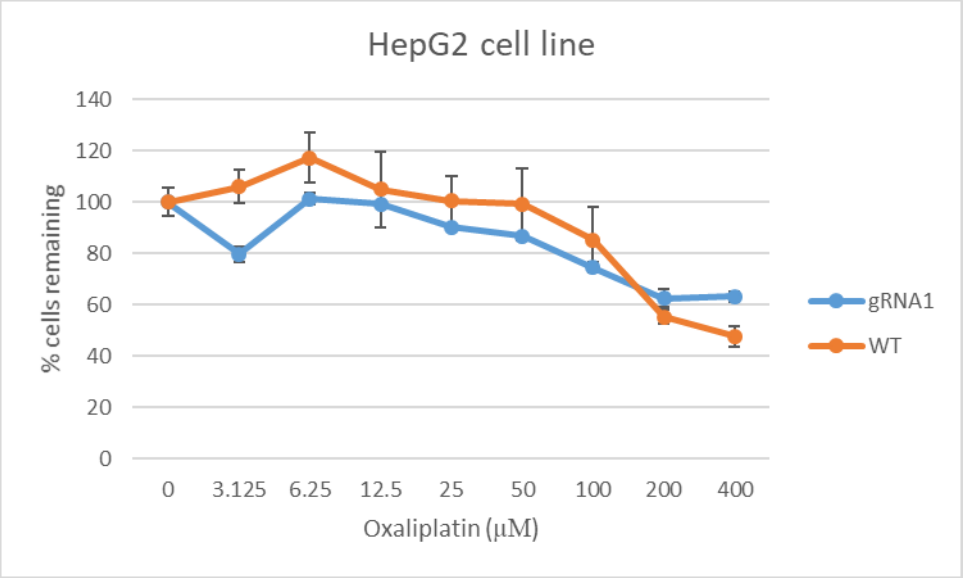

Figure 3. 2 Oxaliplatin induced cytotoxicity in wild-type and ABCC2 gRNA1/Cas9 transfected HepG2 Cells. Data are means ± SD, n = 3.

As shown in Figure 3.2, Wild-type HepG2 cells showed slightly increased proliferation at lower oxaliplatin concentrations. Oxaliplatin showed no apparent cytotoxicity until its concentration exceeds 25 μM. Correspondingly, the ABCC2 gRNA1/Cas9 transfected HepG2 cell line did not show an effect of cell proliferation at low concentration ranges. The concentration cell viability curves appear to fit well with sigmoid model of negative exponential distribution, showing slightly decreased cell viability in the low concentration range followed by relatively steep drop of cell viability. In comparison, the transfected cell line appear to have higher sensitivity at low concentration range, but there is no apparent differences in IC_{50} values between two cell lines. Thus, this may lead us to observe various gene dose effects, as the hybrid cell line exhibits greater resistance to oxaliplatin in MRP2 knockout cells, as it contains at least one surviving gene copy.

3.2 Effect of ABCC2 gRNA2/Cas9 transfection

3.2.1 CDCF accumulation

The difference in transport activity between the knockout HepG2 cell line and its wild-type cell line can be detected using the selected MRP2 substrate. Therefore, CDCF, which is the primary substrate for MRP2, was used as the first step in the present study to identify that the MRP2 gene has been successfully knocked out.

gRNA2-Cas9-transfected knockout cells and wild-type control cells were incubated with 3 µM CDCFDA for 20 minutes in the same experimental environment provided. Then, fluorescence was used to measure the accumulation in the HepG2 cells. The data showed that the CDCF accumulation in the cell line after CRISPR editing increased by 140% ($P < 0.05$) compared with wild type HepG2 cells.

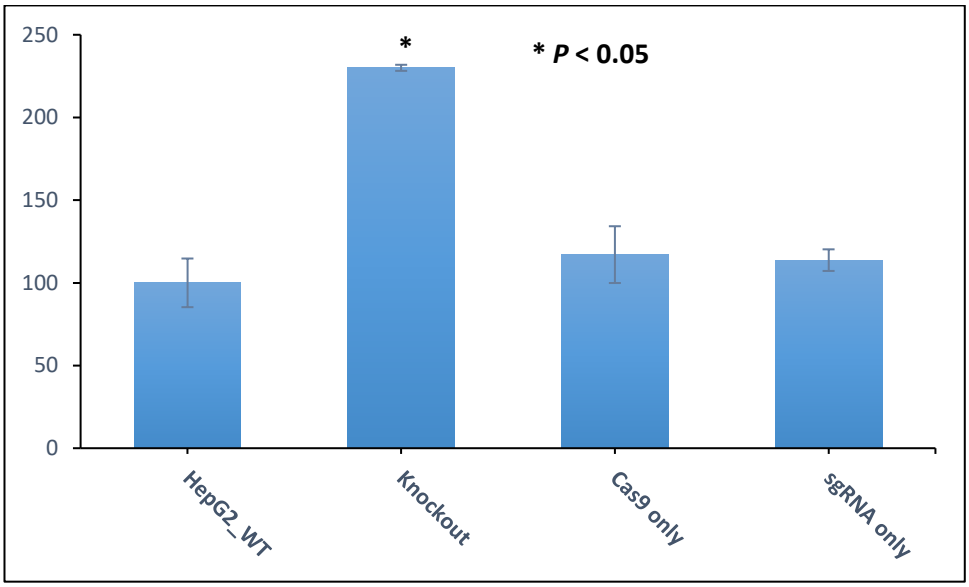

Figure 3. 3Accumulation of CDCF in gRNA2/Cas9 transfected and wild-type HepG2 cells. Data are Means ± SD, n =3. * *P* < 0.05, compared with wild-type, one way ANOVA with Tukey's multiple comparison post-tests.

From the results, knockout cell lines did express less MRP2 protein than wild-type HepG2 cells, as the fluorescent signal in the MRP2 knockout cell line was about 50% higher than the other groups, indicating a reduced ability to transport CDCFDA. (Figure 3.1). We also tested the MRP2 activity of cells that only added CRISPR proteins and

cells that added a unique gRNA. The results showed that there was no significant difference in fluorescence signals compared to wild-type HepG2, which may indicate that their MRP2 expression ability did not change significantly in these cell lines. In short, gene knockout of HepG2 cells has reduced the function of transporting CDCF.

3.2.2 Increased chemosensitivity in HepG2 cells transfected with ABCC2 gRNA2/Cas9

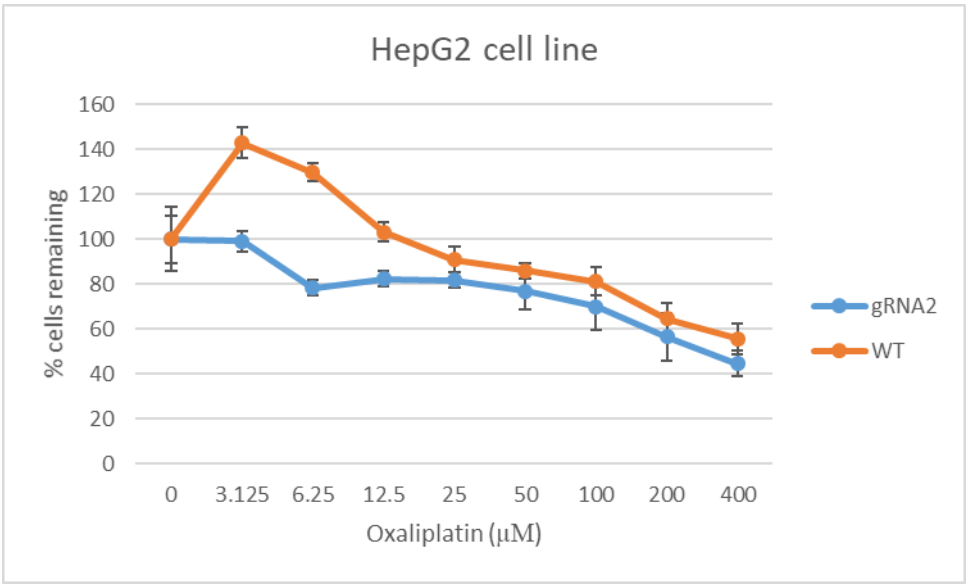

Figure 3. 4 Oxaliplatin induced cytotoxicity in wild-type and ABCC2 gRNA1/Cas9 transfected HepG2 Cells. Data are means ± SD, n = 6. * *P* < 0.05, unpaired t-test.

As shown in Figure 3.1, similar to the gRNA1 group of experiments, wild-type HepG2 cells showed increased proliferation when treated with lower concentrations of oxaliplatin, indicating that drug doses above 12.5 μM showed heightened sensitivity associated with increased doses. In contrast, in the ABCC2 gRNA2/Cas9 transfected cells, the concentration cell viability curves appear to fit well with sigmoid model of negative exponential distribution, showing slightly decreased cell viability in the low concentration range followed by relatively steep drop of cell viability. Oxaliplatin at 12.5 μM has no apparent cytotoxic effects (cell viability 103 ± 7%) in wild-type HepG2 cells but caused significantly ($P < 0.05$) toxic effects in the ABCC2 gRNA2/Cas9

transfected HepG2 cells (cell viability 82 ± 5%). In general, the knockout cell line showed a higher sensitivity to oxaliplatin than the wild type control group.

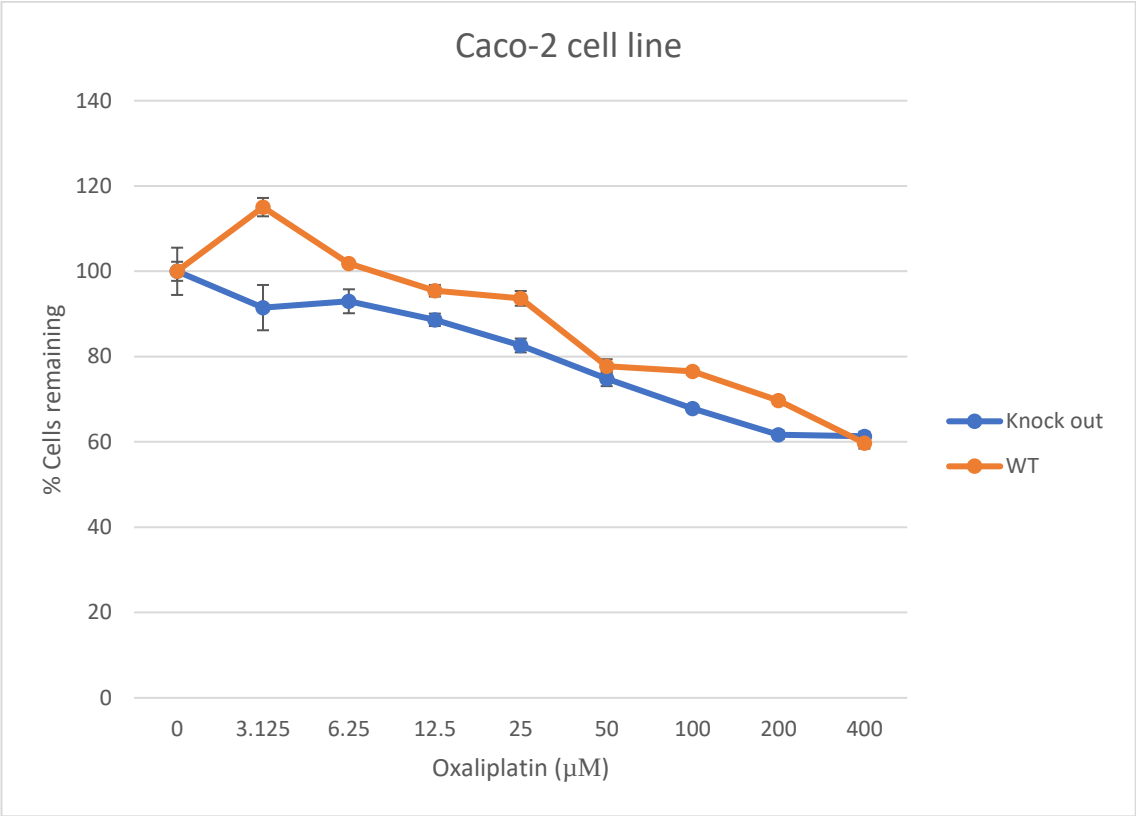

Figure 3. 5 Oxaliplatin induced cytotoxicity in wild-type and ABCC2 gRNA2/Cas9 transfected Caco-2 Cells. Data are means ± SD, n = 6.

In addition, we also compare the chemosensitivity of MRP2 knocked out Caco-2 cell line with wide type Caco-2 cell line. This Caco-2 knock out cell line is also treated with gRNA2. As shown in figure 3.5, the wild-type Caco-2 cell line shows a slight increase in cells proliferation when treated with a concentration of oxaliplatin at 3.125µM. When the concentration of oxaliplatin is higher than 6.25 µM, the cytotoxic effects of oxaliplatin increased by the increase of oxaliplatin concentration in wild type Caco-2 cell line. However, the MRP2 knocked out Caco-2 cell line shows a cytotoxicity effect even at a low oxaliplatin dosage, and this effect is enhanced with the increase of

oxaliplatin concentration. Overall, the knocked-out cell line shows higher chemosensitivity compare with wild-type Caco-2 cell line.

In summary, the Cas-gRNA2 is efficiency in knockout MRP2 protein both in HepG2 and Caco-2 cell line.

3.3 Analyses of gene editing efficiency

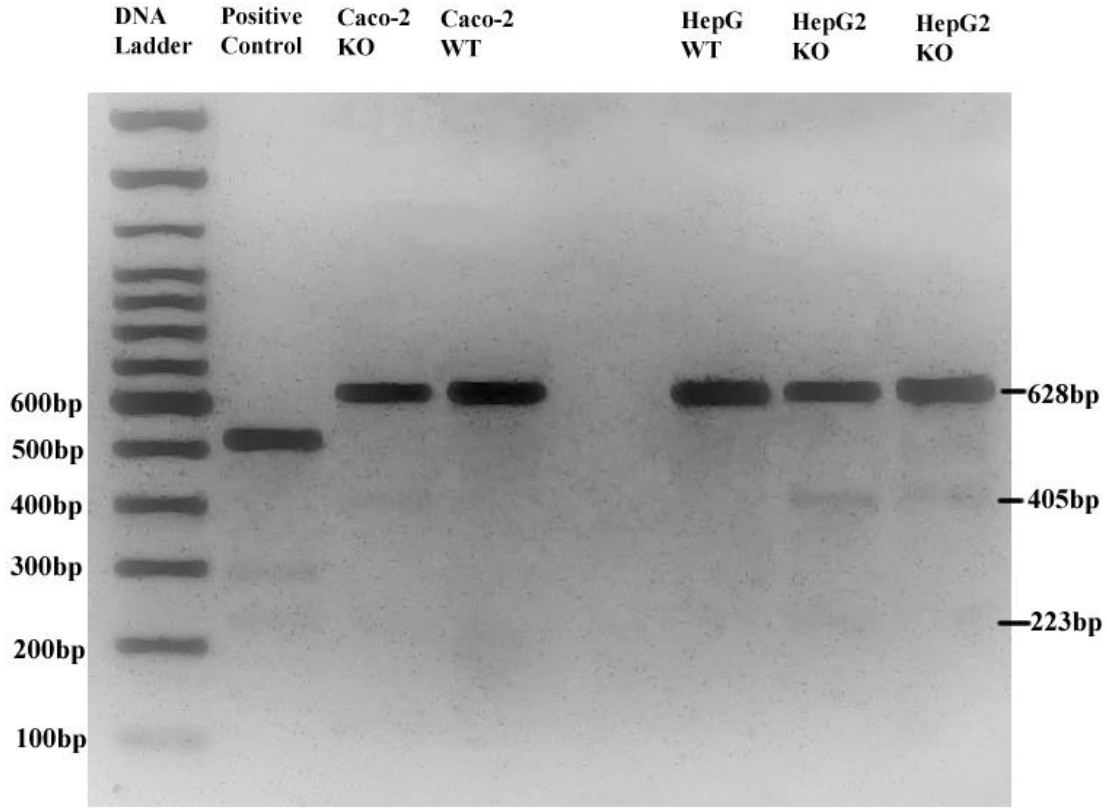

Figure 3. 6 Gel image of Genomic Cleavage Detection Assay using transfected HepG2 and Caco-2 cells.

Further analysis of gene editing efficiency was undertaken by using the GeneArt Genomic Cleavage Detection Kit from Thermo Fisher Scientific, which is T7 endonuclease I based method to quantify how well the genome editing protocol causes insertions and deletions (indels) in the genome of a specific cell line. The CRISPR-Cas9 activity was analyzed using genomic DNA from the designated stably transfected and

wild-type Caco-2 and HepG2 cells. With the preparation of a negative control sample of gene modification, all the samples shown in Figure 3.6 above were PCR amplified using the same set of primers flanking the region of interest. After re-annealing, samples were treated with detection enzyme and run on a 2% agarose gel. The gel image of genomic cleavage detection assay demonstrates that the genomic DNA from gRNA2/Cas9 transfected Caco-2 or HepG2 cell populations shows one parental band (628 bp) and two distinct cleavage bands (405 and 223 bp) confirming gene editing. The estimated genomic cleavage efficiency is 25.2 ± 1.4 % (Mean ± SD) in HepG2 cells based on the duplicate results. Using this T7 endonuclease I based method, on-target genome editing efficiency generated by non-homologous end joining (NHEJ) activity can be quickly and confidently measured.

Chapter 4 Discussion

4.1 Introduction

Cell membrane transporter proteins play pivotal roles in controlling the pharmacokinetic disposition, efficacy and toxicity of oxaliplatin. A recent work identifies a critical potential role for the membrane transporter MRP2 in transport-mediated tumour resistance to oxaliplatin (Myint et al., 2019). A preliminary work with the ABCC2 family member, which encodes multidrug resistance-associated protein 2 (MRP2), has shown in membrane vesicles that MRP2 transports oxaliplatin (Km = 301 μM; Vmax = 2680 pmol Pt/mg protein/10 minutes) (Myint et al., 2015), and that oxaliplatin stimulates MRP2 ATPase activity (EC50 = 8.3 ± 0.7 μM; Hill slope = 2.6) (unpublished). We showed that endogenous over-expression of MRP2 in human HepG2 cancer cells is associated with decreased oxaliplatin accumulation, and that inhibition of MRP2 with siRNA knockdown or myricetin (a model MRP2 inhibitor) reverses those deficits and increases oxaliplatin antitumour cytotoxicity (Myint et al., 2019). A preliminary study of mice bearing tumour xenografts of HepG2 tumour with endogenous MRP2 over-expression demonstrated that inhibiting MRP2 with myricetin caused increased in vivo sensitisation to oxaliplatin antitumour activity with little or no increase in toxicity (Myint et al., 2019). In the analysis of a clinical gene expression dataset, ABCC2 was found to be the only one of 18 oxaliplatin transporter candidate genes with differential tumour expression between CRC patients who did or did not respond to oxaliplatin-based chemotherapy.

The aim of this study was to reduce drug efflux by merely knocking out membrane transport proteins using liposome-delivered CRISPR/Cas9 system, thereby increasing oxaliplatin efficacy in a MRP2 overexpressing in vitro model (HepG2 cells). The first

objective is to confirm on-target effects in CRISPR-Cas9-mediated gene editing, and estimate gene editing efficiency. The MRP2-mediated substrate efflux and tumour resistance has also been assessed in this mixed cell populations. Finally, based on these findings, new insights into genetically edited cancer treatments, how to provide new potential treatment strategies for oxaliplatin-based liver cancer chemotherapy patients, and personalized editing of gene editing systems to further enhance the clinical efficacy.

4.2 Summary of the findings

Our current results reveals the feasibility of application of the liposome delivered CRISPR-Cas9 system to knock out ABCC2 gene in HepG2 cell line.

Two sgRNAs were tested in this paper, one of which showed that the target gene could not be effectively knocked out for unknown reasons. Fortunately, another gRNA plays a role in disabling target genes with a genomic cleavage efficiency of 25% in HepG2 cells. We applied this Cas-gRNA transfection to HepG2 cells and Caco-2 cells, respectively. An increase in CDCF accumulation and an increase in oxaliplatin cytotoxicity were observed for knockout HepG2 cells compared to control wild type. These results demonstrate that the on-target genomic editing in cell line treated by CRISPR-Cas9, and silencing ABCC2 gene by CRISPR-Cas9 does reduced the function of MRP2 protein. Thus, the introduction of gene editing techniques into cancer chemotherapy seeks to overcome current barriers and achieve higher clinical outcomes. These genetic types of genetic tools can identify and perform DNA cleavage and modify tumour genes to help increase their sensitivity to anticancer drugs.

4.3 Gene editing system applied in the improvement of cancer chemotherapy efficiency

Our results provide some support for the notion that the combination of gene-editing activity and chemotherapy acts synergistically to limit tumour cell growth. as mentioned in chapter 1.1.3, despite the surgery and liver transplantation which is feasible in HCC

treatment at an early stage, currently, various HCC chemotherapy strategies including the combinatorial therapy have been investigated for patient with an advanced stage of HCC.

However, most of these chemotherapy strategies have limited efficacy. For example, sorafenib, as the only admitted chemotherapy agent to potentially cure HCC, will eventually trigger the multidrug resistance, with the cure of side effect of doping sorafenib includes diarrhea and weight loss and so on. Other combinational chemotherapy agents include oxaliplatin-based regimens (e.g. FOLFOX4; GEMOX) also present a lack of efficiency. Some oxaliplatin-based regimens such as XELOX (oxaliplatin plus capecitabine) for HCC treatment and have shown impressive results in phase || trials but the ultimate clinical outcomes are unknown at this stage.

Thus, the using of gene editing system in cancer treatment aim to modify the targeted chemoresistant related gene in tumor cells, may enhance chemotherapy efficacy and lead to active killing tumor cell at a lower dosage of chemotherapy agents and in additional help to enhance the clinical outcome of cancer treatment. The choice of the targetable gene for disruption is obviously a crucial component of this type of experimental strategy. For our experiments, MRP2 was chosen because of its well-known association with cytoprotection and its capacity to confer oxaliplatin resistance. An increased level of attention has begun to center on membrane transporter dictating some degree of chemo-resistance, as it appears to have a broader impact on resistance to platinum-based chemotherapeutic approaches than once envisioned. Multiple chemotherapy agents can be the substrates of MRP2, including gemcitabine, which is a wild used nucleoside analogue in the treatment for numbers of cancers (Bugde, et al., 2017). In addition, it has been reported that the chemoresistance in ovarian cervical and lung cancer is mainly derived by the overexpression and enhance of activity of MRP2,

thus using the gene-editing system to restrict the expression of MRP2 protein, can reverse the resistance effect of oxaliplatin in HepG2 cell.

As our experimental results show, the disabled drug intake related gene through gene editing system has increased the cytotoxicity of the anticancer drug by the increase of cellular drug accumulation. Thus, it is feasible to combine chemotherapy with gene editing in the treatment of cancer. However, as out experiment is take in vitro, the in vivo delivery of CRISPR-Cas9 will be another difficult for the various used of gene edting system in clinical trials.

4.4 The off-target effect can affect the gene knockout efficiency

Usually, the gene disruption process was initially started with the design of the CRISPR gRNA. However, due to limited time, we skip this step directly through the purchased gRNA. In this study, two different gRNA sequences (gRNA1 and gRNA2) were purchased from Thermo Fisher, which was designed to be complementary to the target site. We chose different sequences because, as described in Chapter 1.5 above, the choice of gRNA will significantly affect the success rate of gene editing. Selecting the right gRNA is one of the critical factors to avoid possible off-target effects. It turns out that the using of gRNA1 fails to achieve our expected results while gRNA2/Cas9 transfection showed significant on-target ABCC2 gene editing.

We speculate that the gRNA1 fails to knock out the target sequence and leads to an off-target effect. It is important to identifying gRNA as they may disrupt the gene disruption of all known MRP2 isoforms. The results show that when these gRNAs are coupled to the expressed CRISPR protein and applied to the liver cell line, only gRNA2 does cause gene disruption. In this study, genetically engineered cell lines were produced by gRNA2. These results demonstrate the importance of gRNAs providing correct cleavage on target genes. However, even if the second gRNA has successfully

knocked out the target gene, the gelation efficiency of the Genomic Cleavage Detection Assay is limited.

It can be observed from the results that gRNA may be prone to off-target homologous attachment, thereby guiding Cas9 to cleave DNA sequences at undesired sites. Previous studies have identified a number of seed sequences that are less tolerant of gRNA-Cas9 activity mismatches. The definition of the seed sequence varies from researcher to human but is usually interpreted as the first 12 bases at the 3' end of the gRNA sequence, immediately upstream of the PAM. The gRNA-Cas9 system can tolerate 1-3 target mismatches, while 2 mismatches in the gRNA seed sequence can eliminate off-target activity. besides, when the concentrations of gRNA and Cas9 were relatively high, up to 5 mismatched off-target activities were observed. In addition, studies have found that off-target effects depend on the number and location of mismatches between gRNA and target DNA sequences. gRNA-Cas9-induced off-target activity when inserts between target DNA and gRNA was systematically studied and gRNAs with low GC content were less tolerant to mismatches ("Mathematical and computational analysis of CRISPR Cas9 sgRNA off-target homologies," 2016)

In order to avoid the off-target effect, some studies have chosen to use multiple designed gRNA sequences to increase efficiency. In this method, Cas9 is mutated into a nicking enzyme capable of cleaving only one strand of double-stranded DNA. Therefore, in order to obtain DSB on DNA, a pair of sgRNAs must be present at a nearby position, each site being used for the corresponding strand. This dramatically reduces the likelihood of off-target activity, as homologous sequences of both sgRNAs are unlikely to be found at any genomic location. In addition, human genes consisting of multiple exons can produce several protein isoforms during translation. Therefore, the use of various gRNAs is a strategy to improve the efficiency of gene editing.

4.5 MRP2-mediated active transport of oxaliplatin

We choose the membrane transporter MRP2 protein as the gene-editing target as it has been identified by previous studies participating in regulating the cellular accumulation of platinum-based drugs include oxaliplatin. As the member of ABC membrane transporter family, MRP2 shows different from most of the ABCCs subfamily members who expressed on the basolateral membranes; it localized on the apical membrane of polarised cells typically found in hepatocytes and enterocytes (Bugde, et al., 2017). Due to the rich expression of MRP2 typically in the hepatocyte and HepG2 cells, we hypothesize that by disrupting the expression of MRP2 (ABCC2) can enhance cells sensibility towards drugs, and the chemotherapy agents, such as oxaliplatin, would achieve better efficiency with a lower dosage.

Previous studies have demonstrated that MRP2 transports oxaliplatin-derived platinum in an ATP-dependent manner (Myint et al., 2015). MRP2-mediated oxaliplatin-derived platinum transports across the membrane in the presence of ATP. The rate of platinum membrane transport mediated by MRP2 and ATP increased nonlinearly with increasing exposure to oxaliplatin, approaching plateau levels, indicating saturation of the transport process. MRP2 inhibitors (myricetin or MK571) inhibit the accumulation of membrane vesicles in platinum. These findings provide experimental evidence for the interaction of oxaliplatin with the MRP2 protein and transport across the membrane by MRP2-mediated active transport.

MRP2-mediated oxaliplatin transport may be involved in determining the accumulation of oxaliplatin in HepG2 and human liver cancer cell lines. The accumulation of oxaliplatin-derived platinum in HepG2 cells overexpressing MRP2 is reduced, and these findings suggest that MRP2-mediated oxaliplatin or oxaliplatin-derived platinum (Myint, et al., 2019). However, the data was relatively limited and inconclusive about the role of MRP2 in cellular platinum accumulation and cellular resistance to

oxaliplatin. There is no direct evidence that the high expression level of ABCC2 is associated with reduced tumour accumulation of oxaliplatin or oxaliplatin-derived platinum. Further studies are warranted to elucidate the contribution of MRP2 and possibly other ABC transporters on pharmacokinetics and pharmacodynamics of platinum-drugs.

4.6 The implication of CRISPR-Cas9 in personalized therapy of human hepatocellular carcinoma

Functional knockout is of value for the treatment of cancer when CRISPR is turned to clinical use. Its long-term goal is to guide CRISPR activity in tumour cells rather than normal cells, but the achievement of this goal remains a formidable challenge. Our results suggest that gene editing strategies can be designed to disable functional domains that are more abundant in HepG2 cell growth and that have an impact on drug response.

This thesis has provided evidence of the involvement of MRP2 in the transporter-mediated oxaliplatin resistance in human cancer cells. As shown in cytotoxicity results, the knock-out of MRP2 can enhance the chemosensitivity of cancer cells to oxaliplatin. Therefore, MRP2 could possibly be used as the target of chemotherapy to improve the efficacy of the current chemotherapeutic outcome.

However, the resistance mechanism can occur not only in a subset of cancer cell types, and it would appear necessary for the patients to be screened for up-regulation of MRP2 before co-treatment with an MRP2 inhibitor (e.g. myricetin) or ABCC2 knock-out.

As previously mentioned in 1.1.3, HCC is hard to be treated directly by radical treatment as well as to develop new therapeutic alternatives, as this type of carcinoma, as the on only admitted chemotherapy agent sorafenib has a low response rate and eventually will trigger secondary resistance. The current under exploits new chemotherapy agents, including oxaliplatin and its combination treatment, facing the

limitation of their clinical outcome induced by multiple factors. Also, the higher dosage can induce intolerant side effect, which primarily impacts the lives of patients.

Both intrinsic and acquired resistance developed in cancer has an impact on the efficacy of oxaliplatin. There is evidence showing that the reduction of overall drug intake and the increase of drug efflux are the primary factors to induce drug resistance. Also, the patients with recurrence during the course of 5-fluorouracil/leucovorin and oxaliplatin (FOLFOX-4) treatment showed a high expression level of MRP2.

Other studies have observed the correlation of the overexpression of MRP2 with an inadequate response to FOLFOX-4 chemotherapy, which can lead to a shorter survival rate. In contrast, in a small-scale clinical study, the increased expression of MRP2 in cancerous colorectal tissues shows no impact on the overall survival or disease-free survival of the patient (Mirakhorli, et al., 2012). Thus the individual variability of MRP2 genotype and its expression level in tumour cells may be linked to the inter-patient variations in its responses to oxaliplatin therapy and the treatment outcome.

The current study has indicated that MRP2 is responsible for the mediated the active transport of platinum-based drugs includes oxaliplatin. this effect restricts the cellular intake of the overall dosage and reduces the chemosensitivity of hepatocellular cancer cells. this mechanism explains the reported clinical correlation between MRP2 tumor expression with the poor clinical outcomes in patients who have receiving oxaliplatin-based chemotherapy. in this case, itis important to identify the patients exhibiting poor response to oxaliplatin via MRP2-mediated deficient platinum accumulation so that oxaliplatin-based treatment regimens could be tailored to the individual patient depending on their MRP2 status to achieve the most effective treatment outcomes.

For patients with the MRP2-mediated resistance to oxaliplatin due, oxaliplatin dose might need to be adjusted and combined with MRP2 inhibitors such as myricetin to optimize its anti-tumor activity. To select such patients, MRP2 expression levels,

genetic variants of ABCC2, MRP2 functional activity, and oxaliplatin accumulation in tumours may need to be determined.

4.7 Overall conclusions

In summary, this thesis has demonstrated that the CRISPR-Cas9 gene-editing system mediates functional knockout of ABCC2 gene, leading to increased cellular accumulation of a model MRP2 substrate and enhanced oxaliplatin cytotoxicity in HepG2 cells. MRP2 has been shown in the past experiment able to limit the accumulation and growth inhibition of oxaliplatin in certain cancer cell types. Silencing MRP2 by CRISPR/Cas9 in HepG2 cells leads to increased sensitivity to low concentrations of oxaliplatin. Achieving a specific therapeutic effect with the reduction of dose, which is of interest in the clinical use of chemotherapy in various types of cancer. Lowering the dose can reduce drug-induced side effects and improve the quality of life of cancer patients. However, the success rate of editing depends on the choice of sgRNA and the cell line to be applied. Therefore, the efficiency of gene editing depends on a variety of factors and further studies are required to prove the principles in animal models before translation into clinical outcomes. In short, these studies provide in vitro evidence supporting a novel therapeutic strategy that knockout of ABCC2 gene can decrease MRP2 function and increase oxaliplatin chemosensitivity in hepatocellular carcinoma.

References

Adamska, A., & Falasca, M. (2018). ATP-binding cassette transporters in progression and clinical outcome of pancreatic cancer: What is the way forward? *World J Gastroenterol, 24*(29), 3222-3238. https://doi.org/10.3748/wjg.v24.i29.3222

Alexopoulos, L. G., Melas, I. N., Chairakaki, A. D., Saez-Rodriguez, J., & Mitsos, A. (2010). Construction of signaling pathways and identification of drug effects on the liver cancer cell HepG2. *Conf Proc IEEE Eng Med Biol Soc, 2010*, 6717-6720. https://doi.org/10.1109/IEMBS.2010.5626246

Arana, M. R., Tocchetti, G. N., Rigalli, J. P., Mottino, A. D., & Villanueva, S. S. M. (2016). Physiological and pathophysiological factors affecting the expression and activity of the drug transporter MRP2 in intestine. Impact on its function as membrane barrier (Vol. 109, pp. 32-44).

Baliou, S., Adamaki, M., Kyriakopoulos, A. M., Spandidos, D. A., Panayiotidis, M., Christodoulou, I., & Zoumpourlis, V. (2018). CRISPR therapeutic tools for complex genetic disorders and cancer (Review). *Int J Oncol, 53*(2), 443-468. https://doi.org/10.3892/ijo.2018.4434

Bialk, P., Wang, Y., Banas, K., & Kmiec, E. B. (2018). Functional Gene Knockout of NRF2 Increases Chemosensitivity of Human Lung Cancer A549 Cells In Vitro and in a Xenograft Mouse Model. *Mol Ther Oncolytics, 11*, 75-89. https://doi.org/10.1016/j.omto.2018.10.002

Brackman, D. J., & Giacomini, K. M. (2018). Reverse Translational Research of ABCG2 (BCRP) in Human Disease and Drug Response. *Clin Pharmacol Ther, 103*(2), 233-242. https://doi.org/10.1002/cpt.903

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries (Vol. 68, pp. 394-424).

Bricca, R., Goutelle, S., Roux, S., Gagnieu, M. C., Becker, A., Conrad, A., . . . Joint Infection Study, G. (2019). Genetic polymorphisms of ABCB1 (P-glycoprotein) as a covariate influencing daptomycin pharmacokinetics: a population analysis in patients with bone and joint infection. *J Antimicrob Chemother*. https://doi.org/10.1093/jac/dky541

Bugde, P., Biswas, R., Merien, F., Lu, J., Liu, D. X., Chen, M., . . . Li, Y. (2017). The therapeutic potential of targeting ABC transporters to combat multi-drug resistance. Expert Opinion on Therapeutic Targets, 21(5), 511-530. https://doi.org/10.1080/14728222.2017.1310841

Busch, W., & Saier, M. H. (2002). The Transporter Classification (TC) system, 2002 (Vol. 37, pp. 287-337).

Chamberlain, J., Sarfati, D., Cunningham, R., Koea, J., Gurney, J., & Blakely, T. (2013). Incidence and management of hepatocellular carcinoma among Māori and non-Māori New Zealanders. *Australian & New Zealand Journal of Public Health, 37*(6), 520-526. https://doi.org/10.1111/1753-6405.12108

Chavez, A., Tuttle, M., Pruitt, B. W., Ewen-Campen, B., Chari, R., Ter-Ovanesyan, D., . . . Church, G. (2016). Comparison of Cas9 activators in multiple species (Vol. 13, pp. 563-+).

Chen, G., Svirskis, D., Lu, W., Ying, M., Huang, Y., & Wen, J. (2018). N-trimethyl chitosan nanoparticles and CSKSSDYQC peptide: N-trimethyl chitosan conjugates enhance the oral bioavailability of gemcitabine to treat breast cancer. *J Control Release, 277*, 142-153. https://doi.org/10.1016/j.jconrel.2018.03.013

Chen, M., Mao, A., Xu, M., Weng, Q., Mao, J., & Ji, J. (2019). CRISPR-Cas9 for cancer therapy: Opportunities and challenges. *Cancer Letters, 447*, 48-55. https://doi.org/10.1016/j.canlet.2019.01.017

Chen, Y., Zhang, K., Li, Y., Guo, R., Zhang, K., Zhong, G., & He, Q. (2019). Oestrogen-related receptor alpha mediates chemotherapy resistance of osteosarcoma cells via regulation of ABCB1. *J Cell Mol Med, 23*(3), 2115-2124. https://doi.org/10.1111/jcmm.14123

Cheng, M., Cai, W., Huang, W., Chen, Y., Wu, Z., Luo, P., & Yan, W. (2018). Histone deacetylase 6 regulated expression of IL-8 is involved in the doxorubicin (Dox) resistance of osteosarcoma cells via modulating ABCB1 transcription. *Eur J Pharmacol, 840*, 1-8. https://doi.org/10.1016/j.ejphar.2018.09.032

Christopher, A. L., Jason, C. H., James, P. C., & Jerilyn, A. T. (2018). Delivering CRISPR: a review of the challenges and approaches. *Drug Delivery*(1), 1234. https://doi.org/10.1080/10717544.2018.1474964

Cox, D. B. T., Platt, R. J., & Zhang, F. (2015). Therapeutic genome editing: prospects and challenges (Vol. 21, pp. 121-131).

Cyranoski, D. (2016). CRISPR gene-editing tested in a person for the first time. *Nature, 539*(7630), 479. https://doi.org/10.1038/nature.2016.20988

da Motta Girardi, D., Correa, T. S., Crosara Teixeira, M., & Dos Santos Fernandes, G. (2018). Hepatocellular Carcinoma: Review of Targeted and Immune Therapies. *Journal Of Gastrointestinal Cancer, 49*(3), 227-236. https://doi.org/10.1007/s12029-018-0121-4

Dae Won, K., Talati, C., Kim, R., & Kim, D. W. (2017). Hepatocellular carcinoma (HCC): beyond sorafenib-chemotherapy. *Journal of Gastrointestinal Oncology, 8*(2), 256-265. https://doi.org/10.21037/jgo.2016.09.07

de Boussac, H., Orban, T. I., Varady, G., Tihanyi, B., Bacquet, C., Brozik, A., . . . Aranyi, T. (2012). Stimulus-induced expression of the ABCG2 multidrug transporter in HepG2 hepatocarcinoma model cells involves the ERK1/2 cascade and alternative promoters. *Biochem Biophys Res Commun, 426*(2), 172-176. https://doi.org/10.1016/j.bbrc.2012.08.046

De Vera, A. A., Gupta, P., Lei, Z., Liao, D., Narayanan, S., Teng, Q., . . . Chen, Z. S. (2019). Immuno-oncology agent IPI-549 is a modulator of P-glycoprotein (P-gp, MDR1, ABCB1)-mediated multidrug resistance (MDR) in cancer: In vitro and in vivo. *Cancer Lett, 442*, 91-103. https://doi.org/10.1016/j.canlet.2018.10.020

Devkota, S. (2018). The road less traveled: strategies to enhance the frequency of homology-directed repair (HDR) for increased efficiency of CRISPR/Cas-mediated transgenesis (Vol. 51, pp. 437-443).

Drozdzik, M., Busch, D., Lapczuk, J., Muller, J., Ostrowski, M., Kurzawski, M., & Oswald, S. (2018). Protein Abundance of Clinically Relevant Drug Transporters in the Human Liver and Intestine: A Comparative Analysis in Paired Tissue Specimens. *Clin Pharmacol Ther*. https://doi.org/10.1002/cpt.1301

Fletcher, J. I., Haber, M., Henderson, M. J., & Norris, M. D. (2010). ABC transporters in cancer: more than just drug efflux pumps (Vol. 10, pp. 147-156).

Ghosh, D., Venkataramani, P., Nandi, S., & Bhattacharjee, S. (2019). CRISPR-Cas9 a boon or bane: the bumpy road ahead to cancer therapeutics. *Cancer Cell Int, 19*, 12. https://doi.org/10.1186/s12935-019-0726-0

Giacomini, K. M., Huang, S.-M., Tweedie, D. J., Benet, L. Z., Brouwer, K. L. R., Chu, X., . . . International, T. (2010). Membrane transporters in drug development (Vol. 9, pp. 215-236).

Gomaa, A.-I., Khan, S.-A., Toledano, M.-B., Waked, I., & Taylor-Robinson, S.-D. (2008). Hepatocellular carcinoma: epidemiology, risk factors and pathogenesis. *World Journal Of Gastroenterology, 14*(27), 4300-4308.

Gonçalves, E., Behan, F. M., Louzada, S., Arnol, D., Stronach, E. A., Yang, F., . . . Garnett, M. J. (2019). Structural rearrangements generate cell-specific, gene-independent CRISPR-Cas9 loss of fitness effects. *Genome Biology, 20*(1), 27. https://doi.org/10.1186/s13059-019-1637-z

Grazie, M. L., Biagini, M. R., Tarocchi, M., Polvani, S., & Galli, A. (2017). Chemotherapy for hepatocellular carcinoma: The present and the future. *World Journal of Hepatology, 9*(21), 907-920. https://doi.org/10.4254/wjh.v9.i21.907

Guerra, R. M., Bird, G. H., Harvey, E. P., Dharia, N. V., Korshavn, K. J., Prew, M. S., . . . Walensky, L. D. (2018). Precision Targeting of BFL-1/A1 and an ATM Co-dependency in Human Cancer. *Cell Rep, 24*(13), 3393-3403 e3395. https://doi.org/10.1016/j.celrep.2018.08.089

Guo, X., Chitale, P., & Sanjana, N. E. (2017). Target Discovery for Precision Medicine Using High-Throughput Genome Engineering. *Adv Exp Med Biol, 1016*, 123-145. https://doi.org/10.1007/978-3-319-63904-8_7

Han, C. H., Khwaounjoo, P., Kilfoyle, D. H., Hill, A., & McKeage, M. J. (2013). Phase I drug-interaction study of effects of calcium and magnesium infusions on oxaliplatin pharmacokinetics and acute neurotoxicity in colorectal cancer patients (Vol. 13).

Hay, E. A., Khalaf, A. R., Marini, P., Brown, A., Heath, K., Sheppard, D., & MacKenzie, A. (2017). An analysis of possible off-target effects following CAS9/CRISPR targeted deletions of neuropeptide gene enhancers from the mouse genome. *Neuropeptides, 64*, 101-107. https://doi.org/10.1016/j.npep.2016.11.003

Hsiao, S. H., Lusvarghi, S., Huang, Y. H., Ambudkar, S. V., Hsu, S. C., & Wu, C. P. (2019). The FLT3 inhibitor midostaurin selectively resensitizes ABCB1-overexpressing multidrug-resistant cancer cells to conventional chemotherapeutic agents. *Cancer Lett, 445*, 34-44. https://doi.org/10.1016/j.canlet.2019.01.001

Huang, F., Wang, B.-R., & Wang, Y.-G. (2018). Role of autophagy in tumorigenesis, metastasis, targeted therapy and drug resistance of hepatocellular carcinoma. *World Journal Of Gastroenterology, 24*(41), 4643-4651. https://doi.org/10.3748/wjg.v24.i41.4643

Huang, Y., & Sadee, W. (2006). Membrane transporters and channels in chemoresistance and -sensitivity of tumor cells (Vol. 239, pp. 168-182).

Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R., . . . Higgins, C. F. (1990). Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature, 346*(6282), 362-365.

Infantino, V., Dituri, F., Convertini, P., Santarsiero, A., Palmieri, F., Todisco, S., . . . Iacobazzi, V. (2018). Epigenetic upregulation and functional role of the mitochondrial aspartate/glutamate carrier isoform 1 in hepatocellular carcinoma. *Biochim Biophys Acta Mol Basis Dis, 1865*(1), 38-47. https://doi.org/10.1016/j.bbadis.2018.10.018

Jerremalm, E., Wallin, I., & Ehrsson, H. (2009). New Insights Into the Biotransformation and Pharmacokinetics of Oxaliplatin (Vol. 98, pp. 3879-3885).

Ji, N., Yang, Y., Cai, C. Y., Lei, Z. N., Wang, J. Q., Gupta, P., . . . Chen, Z. S. (2019). Selonsertib (GS-4997), an ASK1 inhibitor, antagonizes multidrug resistance in

ABCB1- and ABCG2-overexpressing cancer cells. *Cancer Lett, 440-441*, 82-93. https://doi.org/10.1016/j.canlet.2018.10.007

Joshi, S., & Durden, D. L. (2019). Combinatorial Approach to Improve Cancer Immunotherapy: Rational Drug Design Strategy to Simultaneously Hit Multiple Targets to Kill Tumor Cells and to Activate the Immune System. *J Oncol, 2019*, 5245034. https://doi.org/10.1155/2019/5245034

Karimian, A., Azizian, K., Parsian, H., Rafieian, S., Shafiei-Irannejad, V., Kheyrollah, M., . . . Yousefi, B. (2019). CRISPR/Cas9 technology as a potent molecular tool for gene therapy. *J Cell Physiol*. https://doi.org/10.1002/jcp.27972

Kartal-Yandim, M., Adan-Gokbulut, A., & Baran, Y. (2016). Molecular mechanisms of drug resistance and its reversal in cancer. *Critical Reviews in Biotechnology, 36*(4), 716.

Kimberland, M. L., Hou, W., Alfonso-Pecchio, A., Wilson, S., Rao, Y., Zhang, S., & Lu, Q. (2018). Strategies for controlling CRISPR/Cas9 off-target effects and biological variations in mammalian genome editing experiments. *Journal Of Biotechnology, 284*, 91-101. https://doi.org/10.1016/j.jbiotec.2018.08.007

Kleinstiver, B. P., Prew, M. S., Tsai, S. Q., Topkar, V. V., Nguyen, N. T., Zheng, Z., . . . Joung, J. K. (2015). Engineered CRISPR-Cas9 nucleases with altered PAM specificities (Vol. 523, pp. 481-U249).

Krall, E. B., Wang, B., Munoz, D. M., Ilic, N., Raghavan, S., Niederst, M. J., . . . Hahn, W. C. (2017). KEAP1 loss modulates sensitivity to kinase targeted therapy in lung cancer. *Elife, 6*. https://doi.org/10.7554/eLife.18970

Kurata, M., Yamamoto, K., Moriarity, B. S., Kitagawa, M., & Largaespada, D. A. (2018). CRISPR/Cas9 library screening for drug target discovery. *J Hum Genet, 63*(2), 179-186. https://doi.org/10.1038/s10038-017-0376-9

Lane, T. S., Rempe, C. S., Davitt, J., Staton, M. E., Peng, Y., Soltis, D. E., . . . Stewart, C. N., Jr. (2016). Diversity of ABC transporter genes across the plant kingdom and their potential utility in biotechnology. *BMC Biotechnol, 16*(1), 47. https://doi.org/10.1186/s12896-016-0277-6

Lauretta M.S, C., Simon, L., & Barry H, H. (2004). The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. *European Journal of Pharmaceutical Sciences*(1), 25. https://doi.org/10.1016/j.ejps.2003.07.003

Levine, W. N., Rieger, K., & McCluskey, G. M., 3rd. (2005). Arthroscopic treatment of anterior shoulder instability. *Instr Course Lect, 54*, 87-96.

Liang, X., Potter, J., Kumar, S., Zou, Y., Quintanilla, R., Sridharan, M., . . . Chesnut, J. D. (2015). Rapid and highly efficient mammalian cell engineering via Cas9 protein transfection (Vol. 208, pp. 44-53).

Liu, B., Saber, A., & Haisma, H. J. (2019). CRISPR/Cas9: a powerful tool for identification of new targets for cancer treatment. *Drug Discov Today*. https://doi.org/10.1016/j.drudis.2019.02.011

Liu, B., Yang, H., Pilarsky, C., & Weber, G. F. (2018). The Effect of GPRC5a on the Proliferation, Migration Ability, Chemotherapy Resistance, and Phosphorylation of GSK-3beta in Pancreatic Cancer. *Int J Mol Sci, 19*(7). https://doi.org/10.3390/ijms19071870

Liu, C., Sun, L., Yang, J., Liu, T., Yang, Y., Kim, S. M., . . . Guo, Q. (2018). FSIP1 regulates autophagy in breast cancer. *Proc Natl Acad Sci U S A, 115*(51), 13075-13080. https://doi.org/10.1073/pnas.1809681115

Loise M, F., Peter T, S., & Arlene H, S. (2010). The PD-1 pathway in tolerance and autoimmunity. *Immunological Reviews*(1), 219. https://doi.org/10.1111/j.1600-065X.2010.00923.x

. Mathematical and computational analysis of CRISPR Cas9 sgRNA off-target homologies. (2016) (pp. 449): IEEE.
Martinez-Balibrea, E., Martinez-Cardus, A., Gines, A., Ruiz de Porras, V., Moutinho, C., Layos, L., . . . Abad, A. (2015). Tumor-Related Molecular Mechanisms of Oxaliplatin Resistance (Vol. 14, pp. 1767-1776).
Mendis, S. (2014). *Global status report on noncommunicable diseases 2014* [Electronic document]: Geneva, Switzerland : World Health Organization, [2014]. Retrieved from http://ezproxy.aut.ac.nz/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=cat05020a&AN=aut.b14186469&site=eds-live
http://ezproxy.aut.ac.nz/login?url=http://ebookcentral.proquest.com/lib/AUT/detail.action?docID=2059287
Merino, D., Whittle, J. R., Vaillant, F., Serrano, A., Gong, J. N., Giner, G., . . . Lindeman, G. J. (2017). Synergistic action of the MCL-1 inhibitor S63845 with current therapies in preclinical models of triple-negative and HER2-amplified breast cancer. *Sci Transl Med, 9*(401). https://doi.org/10.1126/scitranslmed.aam7049
Mirakhorli, M., Shayanfar, N., Rahman, S. A., Rosli, R., Abdullah, S., & Khoshzaban, A. (2012). Lack of association between expression of MRP2 and early relapse of colorectal cancer in patients receiving FOLFOX-4 chemotherapy. Oncology letters, 4(5), 893-897. https://doi.org/10.3892/ol.2012.889
Mollanoori, H., Shahraki, H., Rahmati, Y., & Teimourian, S. (2018). CRISPR/Cas9 and CAR-T cell, collaboration of two revolutionary technologies in cancer immunotherapy, an instruction for successful cancer treatment. *Hum Immunol, 79*(12), 876-882. https://doi.org/10.1016/j.humimm.2018.09.007
Myint, K., Biswas, R., Li, Y., Jong, N., Jamieson, S., Liu, J., . . . McKeage, M. (2019). Identification of MRP2 as a targetable factor limiting oxaliplatin accumulation and response in gastrointestinal cancer. Scientific Reports, 9(1), 2245. https://doi.org/10.1038/s41598-019-38667-8
Oostendorp, R. L., Beijnen, J. H., & Schellens, J. H. M. (2009). The biological and clinical role of drug transporters at the intestinal barrier (Vol. 35, pp. 137-147).
Partridge, E. C., Watkins, T. A., & Mendenhall, E. M. (2016). Every transcription factor deserves its map: Scaling up epitope tagging of proteins to bypass antibody problems. *Bioessays, 38*(8), 801-811. https://doi.org/10.1002/bies.201600028
Pyr dit Ruys, S., Delaive, E., Demazy, C., Dieu, M., Raes, M., & Michiels, C. (2009). Identification of DH IC-2 as a HIF-1 independent protein involved in the adaptive response to hypoxia in tumor cells: A putative role in metastasis. *Biochim Biophys Acta, 1793*(11), 1676-1690. https://doi.org/10.1016/j.bbamcr.2009.09.003
Ren, J., Liu, X., Fang, C., Jiang, S., June, C. H., & Zhao, Y. (2017). Multiplex Genome Editing to Generate Universal CAR T Cells Resistant to PD1 Inhibition. *Clin Cancer Res, 23*(9), 2255-2266. https://doi.org/10.1158/1078-0432.CCR-16-1300
Ren, J., Zhang, X., Liu, X., Fang, C., Jiang, S., June, C. H., & Zhao, Y. (2017). A versatile system for rapid multiplex genome-edited CAR T cell generation. *Oncotarget, 8*(10), 17002-17011. https://doi.org/10.18632/oncotarget.15218
Robey R W, Pluchino K M, Hall M D, Fojo A T, Bates S E, and Gottesman M M. Revisiting the role of ABC transporters in multidrug-resistant cancer. Nat. Rev. Cancer, 2018.
Rodriguez-Rodriguez, D. R., Ramirez-Solis, R., Garza-Elizondo, M. A., Garza-Rodriguez, M. L., & Barrera-Saldana, H. A. (2019). Genome editing: A

perspective on the application of CRISPR/Cas9 to study human diseases (Review). *Int J Mol Med*. https://doi.org/10.3892/ijmm.2019.4112

Saber, A., Liu, B., Ebrahimi, P., & Haisma, H. J. (2019). CRISPR/Cas9 for overcoming drug resistance in solid tumors. *Daru*. https://doi.org/10.1007/s40199-019-00240-z

Sampat, K. R., & O'Neil, B. (2013). Antiangiogenic Therapies for Advanced Hepatocellular Carcinoma (Vol. 18, pp. 430-438).

Shao, J., Glorieux, C., Liao, J., Chen, P., Lu, W., Liang, Z., . . . Huang, P. (2018). Impact of Nrf2 on tumour growth and drug sensitivity in oncogenic K-ras-transformed cells in vitro and in vivo. *Free Radic Res, 52*(6), 661-671. https://doi.org/10.1080/10715762.2018.1462494

Slyskova, J., Sabatella, M., Ribeiro-Silva, C., Stok, C., Theil, A. F., Vermeulen, W., & Lans, H. (2018). Base and nucleotide excision repair facilitate resolution of platinum drugs-induced transcription blockage. *Nucleic Acids Res, 46*(18), 9537-9549. https://doi.org/10.1093/nar/gky764

Stein, A., & Arnold, D. (2012). Oxaliplatin: a review of approved uses (Vol. 13, pp. 125-137).

Szakács, G., Paterson, J. K., Ludwig, J. A., Booth-Genthe, C., Gottesman, M. M., & Szakács, G. (2006). Targeting multidrug resistance in cancer. *Nature Reviews Drug Discovery, 5*(3), 219.

Szakács, G., Annereau, J.-P., Lababidi, S., Shankavaram, U., Arciello, A., Bussey, K. J., . . . Gottesman, M. M. (2004). Predicting drug sensitivity and resistance: Profiling ABC transporter genes in cancer cells. Cancer Cell, 6(2), 129-137. https://doi.org/https://doi.org/10.1016/j.ccr.2004.06.026

Thu, K. L., Silvester, J., Elliott, M. J., Ba-Alawi, W., Duncan, M. H., Elia, A. C., . . . Cescon, D. W. (2018). Disruption of the anaphase-promoting complex confers resistance to TTK inhibitors in triple-negative breast cancer. *Proc Natl Acad Sci U S A, 115*(7), E1570-E1577. https://doi.org/10.1073/pnas.1719577115

Vasiliou, V., Vasiliou, K., & Nebert Daniel, W. (2009). Human ATP-binding cassette (ABC) transporter family. *Human Genomics, Vol 3, Iss 3, Pp 281-290 (2009)*(3), 281. https://doi.org/10.1186/1479-7364-3-3-281

Xia, A. L., He, Q. F., Wang, J. C., Zhu, J., Sha, Y. Q., Sun, B., & Lu, X. J. (2019). Applications and advances of CRISPR-Cas9 in cancer immunotherapy. *J Med Genet, 56*(1), 4-9. https://doi.org/10.1136/jmedgenet-2018-105422

Yoshihisa, S., Toshiharu, H., & Yuichi, S. (2006). Transporters as a determinant of drug clearance and tissue distribution. *European Journal of Pharmaceutical Sciences*(5), 425. https://doi.org/10.1016/j.ejps.2005.12.003

Zhang, C., Liu, J., Zhong, J. F., & Zhang, X. (2017). Engineering CAR-T cells. *Biomark Res, 5*, 22. https://doi.org/10.1186/s40364-017-0102-y

Zhang, H., Song, H., Yuan, R., Zhang, X., Yu, H., Zhao, Y., & Jiang, T. (2018). Polyene phosphatidylcholine overcomes oxaliplatin resistance in human gastric cancer BGC823 cells. *Biochem Biophys Res Commun, 497*(1), 108-114. https://doi.org/10.1016/j.bbrc.2018.02.033