THE MECHANISTIC STUDY OF ACQUIRED RESISTANCE TO APATINIB IN HEPATOCELLULAR CARCINOMA

CHOW LAM

M.Phil.

The Hong Kong Polytechnic University

2020

The Hong Kong Polytechnic University

Department of Applied Biology and Chemical Technology

The Mechanistic Study of Acquired Resistance to Apatinib in Hepatocellular Carcinoma

Chow Lam

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Philosophy

August 2020

Certificate of Originality

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it reproduces no material previously published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

……. (Signed)

Chow Lam

…….....(Name of student)

Abstract of thesis entitled

The Mechanistic Study of Acquired Resistance to Apatinib in Hepatocellular Carcinoma

submitted by

Chow Lam

for the degree of Master of Philosophy

at The Hong Kong Polytechnic University

August 2020

Hepatocellular carcinoma (HCC) is one of common cause of cancer mortality worldwide. The treatment for HCC has entered a new era with the development of molecular-targeted therapies since the breakthrough discovery of sorafenib in 2007. Sorafenib, a multi-kinase inhibitor, blocks tumor cell proliferation by specifically targeting multiple growth factor pathways, and it exerts an anti-angiogenic effect. However, the survival benefit of sorafenib treatment is unsatisfactory due to development of acquired drug resistance. Recently, increasing body of evidence has shown that apatinib, a novel inhibitor of vascular endothelial growth factor receptor-2 (VEGFR2), has good safety and potent therapeutic effect on Chinese patients with advanced HCC. Due to these encouraging results, apatinib has received approval for treatment of advanced HCC in China. Similar to other tyrosine kinase inhibitors, HCC cells also acquire drug resistance to apatinib, which hinders its efficacy. To increase the treatment efficacy of apatinib for HCC patients, combination therapy targeting multiple signaling pathways may serve as a better treatment option by potentially circumventing drug resistance.

In this study, we first established apatinib-resistant HCC cells *in vitro* through stepwise increase in apatinib dosage for 6 months. We afterward have characterized the resistant nature of these cells by morphological analysis and functional assays like Annexin V staining, migration and invasion assays and Western blotting. After verification, we have identified the

molecules/pathways associated with the resistance by comparing the genetic profiles between apatinib-resistant HCC cells and their mock counterparts by RNA sequencing analysis. Upon analysis, we discovered that CPY1A1, a family member of cytochrome P450 superfamily of enzymes, was commonly upregulated in apatinib-resistant HCC cells with the highest fold-change in expression levels, which was further confirmed by qPCR and western blot analyses. Consistently, CPY1A1 enzymatic activity was also found to be upregulated in the resistant cells.

We have successfully repressed the expression and activity of CYP1A1 in HCC cells through lentiviral-based gene knockdown. The CYP1A1-knockdown cells showed increased sensitivity towards apatinib treatment in Annexin V staining. To further understand the molecular mechanism of how CYP1A1 sensitizes the effect of apatinib, RNA sequencing analysis was performed to compare the genetic profiles between CYP1A1-knockdown cells and their non-target control (NTC) counterparts. Upon Ingenuity Pathway Analysis (IPA) and Gene Set Enrichment Analysis (GSEA) analyses, we found that knockdown of CYP1A1 suppressed xenobiotic and bile acid metabolism, RhoGDI, SPINK1 and calcium signaling pathways. All in all, our study not only showed the potential tumor suppressive effect of apatinib on HCC cells, but also provides a mechanistic insight for the development of acquired apatinib resistance in HCC cells. In conclusion, targeting CYP1A1 and its downstream pathways in combination with apatinib may be the potential therapeutic regimen for treatment of advanced HCC patients.

(445 words)

Acknowledgement

I would like to express my sincere gratitude to my supervisor Dr. Terence Lee for providing me an opportunity to pursue my M.Phil. degree and the continuous support of my entire study and research, and for his greatest patience, motivation, enthusiasm, and immerse knowledge. Dr. Terence Lee keens on teaching and learning and cultivates students' ingenuity, as well as encourages them to have a global vision on technological advances in molecular and cancer biology. He has educated me on how to plan and execute a research project in an appropriate way and on the importance of communication, attitude, and responsibility in being a professional scientific researcher. His guidance helped me in all the time of research and writing of this thesis.

I would also like to express my appreciation to Dr. Carmen Leung, Dr. Nicole Ho, Dr. Doris Leung, and Ms. Martina Lei for offering me generous support and precious advice in my project when I was facing difficulties. I would like to thank my fellow teammates Ms. Katherine Chung and Mr. Etienne Mok for all the joy and happiness we have shared during my study period.

Last but not the least, I would like to thank my family and friends for your enormous support, understanding, and kindness throughout my life. Your help always saves me from frustration and confusion.

Table of Contents

List of Figures

List of Tables

List of Abbreviation

ACTN4	α-Actinin 4
AKT	Protein kinase B
AMPK	5' Adenosine monophosphate-activated protein kinase
ANF	α-Naphthoflavone
AR	Apatinib resistance
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
AXL	Anexelekto receptor tyrosine kinases
BAK	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
BCL-2	B-cell lymphoma 2
BCLC	Barcelona Clinic Liver Cancer
CFDA	China Food and Drug Administration
CSC	Cancer stem cells
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
DFG	Asp-Phe-Gly motif
DMSO	Dimethyl sulfoxide
DUSP1	Dual specificity protein phosphatase 1
ECGF1	Endothelial cell growth factor 1
EMSA	Electrophoretic mobility shift assays
EMT	Epithelial-to-mesenchymal transition
ERK1/2	Extracellular signal-regulated kinase 1/2
FATS	Fragile-site associated tumor suppressor
FITC	Fluorescein isothiocyanate
FLT-3	Fms-like receptor tyrosine kinase 3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSEA	Gene Set Enrichment Analysis
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IC50	Half maximal inhibitory concentration
IGF1	Insulin-like growth factor 1
IHC	Immunohistochemistry
JCRB	Japanese Cancer Research Bank
MAPK	Mitogen-activated protein kinase
MOMP	Mitochondrial outer membrane permeabilization
MSigDB	Molecular Signatures Database
MTORC1	Mammalian target of rapamycin complex 1
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NCBI	National Center for Biotechnology Information
NFKB	Nuclear factor-kappa B
NRE1	Negative regulatory element 1
NRF	Negative regulatory factor
NSCLC	Non-small-cell lung carcinoma
NTC	Non-target control
OD	Optical density
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
PAH	Polycyclic aryl hydrocarbons
qPCR	Real-time polymerase chain reaction
PFA	Paraformaldehyde
PI	Propidium iodide
PTEN	Phosphatase and tensin homolog deleted from chromosome 10
PVDF	Polyvinylidene fluoride
RIN	RNA integrity number
RIPA	Radioimmunoprecipitation assay
SERT	Serotonin transporter
SNP	Single nucleotide polymorphism
TACE	Transarterial chemoembolization
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TKI	Tyrosine kinase inhibitor
TPM	Transcripts per million
USFDA	United State Food and Drug Administration
VEGFR2	Vascular endothelial growth factor receptor 2
WHO	World Health Organization

CHAPTER 1

INTRODUCTION TO HEPATOCELLULAR CARCINOMA AND APATINIB

Section 1.1 Epidemiology and Etiology of Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is a lethal disease and is one of the common causes of mortality worldwide. The global distribution of HCC varies by region, sex, and etiology. Liver cancer is a major health problem in developing countries. The highest age adjusted incidence rates (>20 per 100,000) are recorded in East Asia and sub-Saharan Africa, which accounts for 82% of liver cancer cases worldwide (R. X. Zhu, Seto, Lai, & Yuen, 2016). In particular, over 50% of all new HCC cases worldwide are reported from China (Zheng et al., 2018). A medium-high incidence rate is recorded in Southern Europe, whereas low-incidence areas (<5 per 100,000) are recorded in South and Central America, and the rest of Europe. Globally, liver cancer is more prevalent in men than in women, with the male to female ratio ranging between 2:1 and 4:1 and the difference being much greater in high-risk regions (Liu et al., 2017). These variations of distribution patterns are likely related to the prevalence of hepatitis virus infection in the population and the existence of other risk factors.

HCC is a complex disease entity with multiple possible etiologies and is associated with a number of risk factors and cofactors. Approximately 70-90% of patients with HCC have an available case history of chronic liver disease and cirrhosis, with major risk factors including chronic infection with hepatitis B virus (HBV), hepatitis C virus (HCV), alcoholic liver disease, nonalcoholic fatty liver disease (NAFLD), and nonalcoholic steatohepatitis (NASH). Additional risk factors for HCC include aflatoxin exposure, diabetes, obesity, hemochromatosis, and metabolic disorders (Sanyal, Yoon, & Lencioni, 2010).

Chronic liver diseases result in continuous inflammation and damage to the liver, fibrosis, and cirrhosis of the liver. Cirrhosis is a pathological condition in which the liver cannot function properly due to long-term and continuous damage to the liver causing irreversible scarring on it. In turn, cirrhotic liver frequently develops HCC as the disease progresses (Ramakrishna et al., 2013). The pathogenesis of HCC is a multistep process involving the progressive accumulation of molecular alterations pinpointing different molecular and cellular events, such as alternations in the genes related to the p53-RB pathway, Wnt signaling pathway, and chromatin-remodeling pathways, which have been recently identified as significant driver-gene candidates in HCC patients (Kanda, Goto, Hirotsu, Moriyama, & Omata, 2019).

1.1.1. Chronic HBV infection

Chronic infection with HBV has a close association with HCC. It is estimated that more than 50% of HCC cases overall are attributable to chronic liver diseases such as cirrhosis caused by chronic HBV infection. Moreover, it is responsible for 75-90% of HCC cases in HBV endemic or hyperendemic regions (R. X. Zhu et al., 2016). Globally, 270 million people are infected with chronic HBV, which is almost 5% of the world's populations (Nayagam et al., 2016). The majority of patients are found in the HCC high-risk regions of Asia-Pacific and sub-Saharan Africa. Currently, 75% of chronic HBV patients are found in Asia where chronic HBV infection accounts for 80% of all newly diagnosed HCC.

1.1.2. Chronic HCV infection

Chronic HCV infection is the leading cause of HCC in the Western countries and Japan. It is estimated that approximately 180 million people worldwide are infected

with chronic HCV, which accounts for about 3% of the world population (Lemoine, Nayagam, & Thursz, 2013). HCV-infected patients have a risk of HCC increased by 15- to 20-fold. In Japan, the HCV prevalence is around 3% and an estimated 85% of patients with advanced HCC have a case history of chronic HCV infection. In contrast, the US has a lower HCV prevalence of 1.8% of its population, with approximately 60% of patients with HCC being infected with chronic HCV (Axley, Ahmed, Ravi, & Singal, 2018).

1.1.3. Alcoholism

Alcoholism is well recognized as one of the causes of chronic liver disease and HCC. According to the World Health Organization (WHO), liver disease is responsible for 70% of directly recorded mortality from alcohol, and alcohol causes around 80% of deaths from liver disease (Stickel, Datz, Hampe, & Bataller, 2017). Liver function is responsible for most alcohol metabolism through three metabolic systems including the microsomal ethanol oxidizing system, cytosolic alcohol dehydrogenase, and catalase. Severe alcohol intake or chronic alcoholism overwhelming the capacity of the liver can lead to liver damage through endotoxins, oxidative stress, and inflammation, enhancing the development of cirrhosis and HCC (Testino, Leone, & Borro, 2014).

1.1.4. NAFLD and NASH

Increasing evidence has linked obesity, type 2 diabetes mellitus, and metabolic syndrome closely to the risk and mortality of various cancers including NAFLD-related HCC (Z. M. Younossi et al., 2011). NAFLD is the conditions where excessive fat is built up in the liver with insulin resistance due to causes other than alcohol use, according to the 11th revision of the International Classification of Diseases. NASH is

the major type of NAFLD in which inflammation and cell damage occur in the fatty liver, in turn resulting in liver cirrhosis and/or HCC. It is estimated that the current prevalence rate of NAFLD has reached 24-30% of population worldwide (Z. Younossi et al., 2018). The incidence of NAFLD-related HCC is rising globally in parallel with the prevalence of obesity and diabetes.

Section 1.2 Current Treatments and Challenges

Therapeutic options for HCC patients are indicated by a cancer staging system. A stage of cancer refers to the severity of the disease, such as the size and the spread of the tumor, providing physicians with information about the disease details to determine suitable treatments for the patients. There are many staging systems including the TNM staging system and the Barcelona Clinic Liver Cancer (BCLC) classification.

The TNM system is the most widely used cancer staging system in hospitals and medical centers as the main method for cancer reporting in general. The TNM system describes the cancer by assessing the size and extent of the primary tumor, the number of nearby lymph nodes that become cancerous, and whether the tumor has metastasized to distinct parts of the body.

More specifically, the Barcelona clinic liver cancer classification creates a set of criteria to guide the treatments for patients with HCC. It has been commonly implemented as a guideline in hospitals and academics worldwide. The BCLC staging system is developed from the results obtained in the setting of multiple cohort studies and randomized controlled trials (Llovet, Bru, & Bruix, 1999). The BCLC is the first system to recommend evidence-based clinical treatments for each patient at different

stages, such as curative treatments for early stages as well as palliative treatments for advanced stages (Figure 1).

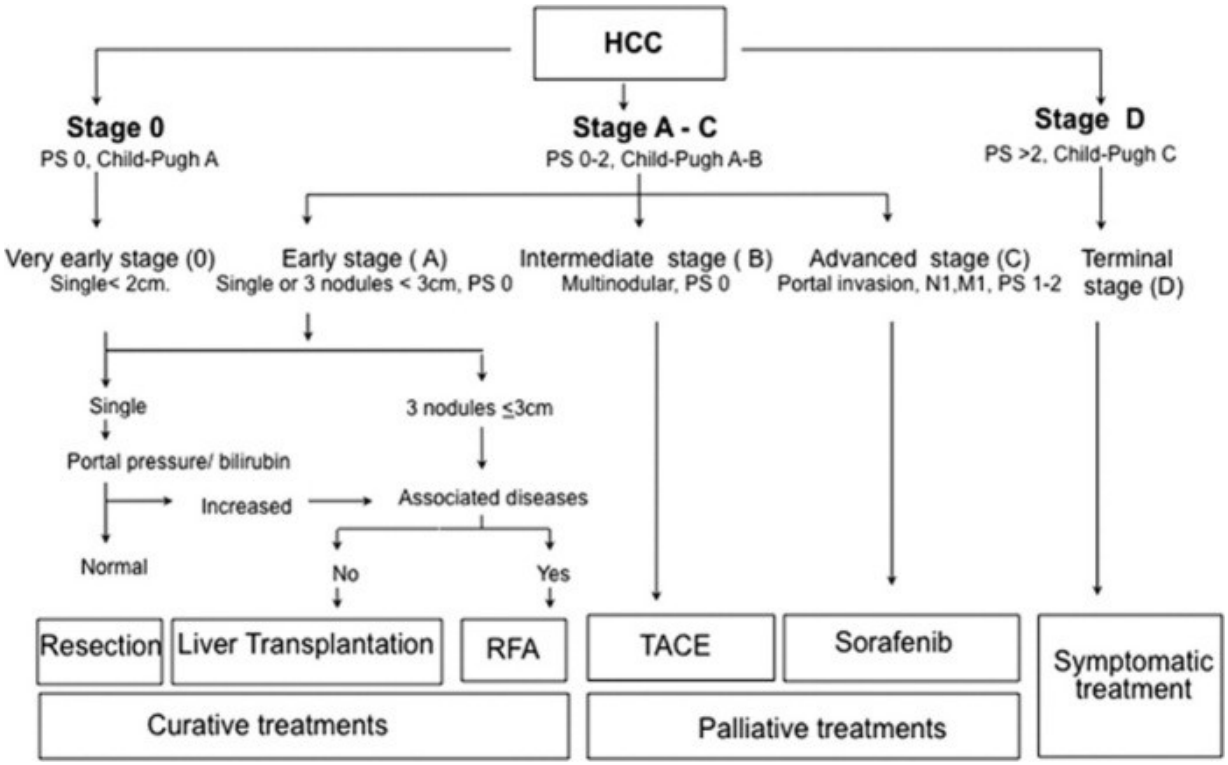

Figure 1. Therapeutic options for HCC patients are indicated by a cancer staging system. The BCLC staging system to guide suitable treatments for patients with HCC (Bruix, Sherman, & American Association for the Study of Liver, 2011).

1.2.1. Liver transplantation / Surgery

The absence of proper curative treatments has encouraged extensive research against HCC to develop new therapeutic strategies. Until now, surgical methods including liver resection, transplantation, and radiofrequency ablation are considered as the only curative treatments for patients with HCC at early stage but not at advanced stage, because advanced HCC frequently has portal vein tumor invasion and extrahepatic metastasis that the tumors cannot be removed solely by surgery. However, owing to the fact that most patients do not have any noticeable symptoms associated with HCC during early stage, diagnosis of HCC at early stage is usually challenging, and thus the majority of HCC patients are diagnosed at the advanced or terminal stage. In addition, there is still no curative treatment for patients with advanced and unresectable HCC.

1.2.2. Chemotherapy

In case a patient cannot be treated surgically, palliative treatments are given to the patient according to the BCLC staging system. Commonly, transarterial chemoembolization (TACE) and sorafenib are used in the treatments of HCC at intermediate and advanced stages respectively, but the TACE has limited efficacy in achieving objective response, with a high risk of complications which can lead to death in some cases. It is not recommended for patients with advanced HCC because this intervention is precluded by portal vein thrombosis or tumor infiltrating blood vessels, and thereby the patients can only receive sorafenib treatment as a palliative care until terminal stage.

1.2.3. Molecularly targeted therapy

Sorafenib is an orally active multi-kinase inhibitor drug that potently targets multiple intracellular kinases such as RAF kinases and cell surface receptor tyrosine kinases like vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptor beta (PDGFRβ), KIT, RET, and FLT-3 (Llovet et al., 2008). Sorafenib has been approved by the United States Food and Drug Administration (USFDA) since 2007 and is currently used as a first-line standard of care for patients with advanced and unresectable HCC. However, based on the fact that it confers limited survival benefit, sorafenib is only considered as a palliative treatment for patients with advanced HCC.

Although sorafenib is a great breakthrough in the development of therapeutic interventions against HCC, the limitations of sorafenib treatment, mostly due to drug resistance frequently resulting in tumor recurrence as well as treatment cost and severe adverse events that require treatment dropout, have prompted current research to explore possibilities of developing novel drug interventions against the disease. In 2017, regorafenib, which is a derivative from sorafenib by addition of a fluorine atom in the central phenyl ring and has stronger potency than sorafenib (Bruix et al., 2017), was approved as a second-line treatment for patients with advanced HCC after failure of sorafenib treatment. In 2018, lenvatinib, a multi-kinase inhibitor against the VEGFRs, were approved as a first-line drug for patients with unresectable HCC (Kudo et al., 2018). The VEGFRs are closely associated with tumor angiogenesis commonly observed in HCC which is well characterized with hypervasculature. Other recently USFDA-approved targeted drugs against advanced HCC include cabozantinib, a multi-kinase inhibitor targeting VEGFR2, c-Met, AXL, and RET (Abou-Alfa et al., 2018).

1.2.4. Immune checkpoint therapy

Recent development of cancer immunotherapy has revealed a new era of drug treatment for HCC. Nivolumab is a fully human immunoglobulin G4 Programmed Death-1 (PD-1) immune checkpoint inhibitor antibody that effectively blocks the PD-1 and promotes antitumor immunity (El-Khoueiry et al., 2017). It was approved in 2017 as a second-line treatment for advanced HCC. The PD-1 has been found to facilitate cancer cells to escape from being killed by immune cells like T cells. Two other fully human monoclonal antibody drugs involve ramucirumab, an anti-VEGFR2 antibody (Zhuang et al., 2019), and pembrolizumab, an anti-PD-1 antibody (A. X. Zhu et al., 2018). In the future, more novel drug interventions are expected to be approved for advanced HCC.

Section 1.3 Apatinib as a Novel Drug for HCC

In recent years, advanced understanding over the molecular mechanisms underpinning tumor initiation and progression have created opportunities for developing novel drug interventions. Apatinib, also known as YN968D1 and rivoceranib, is a novel anti-angiogenic agent targeting the vascular endothelial growth factor receptor 2 (VEGFR2), with encouraging preclinical and clinical performance in treatments of various types of solid tumor. It has been approved by the China Food and Drug Administration (CFDA) and marketed in China since 2014 as a second-line drug treatment for patients with advanced, metastatic gastric cancer. In addition, it is also undergoing phase II/III clinical trials in China for treatments of other types of cancer including lung cancer (Y. Xu et al., 2019), breast cancer (NCT03535961), ovarian cancer (Lan et al., 2018), osteosarcoma (Xie et al., 2019), colorectal cancer (X. Chen et al., 2019), pancreatic cancer (NCT02726854), and hepatocellular carcinoma (Qin,

2014). Therefore, apatinib has potential antitumor activity across a broad range of advanced solid tumors.

Apatinib is a novel VEGFR2 inhibitor that possesses the highest selectivity as compared with other multi-kinase inhibitors involving sorafenib. It effectively blocks the migration and proliferation of vascular endothelial cells, reduces tumor microvessel density, and suppresses tumor growth. Recently, increasing number of clinical trials have proved that apatinib has good safety and potent therapeutic effect on patients with advanced HCC. These encouraging results have collectively proposed that apatinib may be possibly used as a subsequent line of treatment for advanced HCC. Therefore, this study aims to provide evidence to support the stance that apatinib can be used as a potent drug for treating HCC. In addition, drug resistance is a great concern during the development of drug-based therapies, which can result in treatment difficulties and failure. Currently, there is insufficient knowledge about the resistance to apatinib in HCC, and it is of paramount importance to study the molecular mechanism underlying the apatinib resistance in HCC, and thus this project will provide evidence related to apatinib resistance in HCC in order to ultimately explore possibilities of developing further therapeutic implications such as combination therapy.

1.3.1. Structure and biochemistry

Apatinib is an orally bioavailable, small molecule tyrosine kinase inhibitor (TKI). This multi-kinase drug has a molecular formula of $C_{24}H_{23}N_5O$ with a molecular weight of 397.48 g mol^{-1}. Apatinib is commercially available in the form of mesylate salts, having a molecular formula of $C_{25}H_{27}N_5O_4S$ with a molecular weight of 493.58

g mol^{-1}. Apatinib mesylate has pale yellow solid appearance and is soluble in organic solvents such as dimethyl sulfoxide (DMSO) and ethanol.

Intriguingly, apatinib has a molecular structure similar to vatalanib and AAL-993 **(Figure 2)**. Vatalanib is an anti-angiogenic and anti-neoplastic anilinophthalazine that has been reported in early studies to selectively inhibits VEGFRs via binding to the cytoplasmic protein kinase domain of these receptors. In addition, vatalanib is comprised of a core phthalazine moiety which grants it promising anti-cancer potency. The potent VEGFR inhibitor AAL-993 is designed from vatalanib by scaffold morphing and subsequent lead optimization. AAL-993 contains a core anthranilamide moiety that conformationally mimics the phthalazine core of vatalanib by forming a pseudo-bicyclic ring through intramolecular hydrogen bonding between the amine group and the carboxylic group, exerting inhibition of VEGFR2 activity (Furet et al., 2003). Intriguingly, apatinib possesses an anthranilamide-like moiety as its core structure with substitution of a carbon atom with a nitrogen atom in the aromatic ring. The anthranilamide-like moiety is considered responsible for the potent antitumor property of apatinib. The substitution however does not alter the formation of the pseudo-bicyclic ring mimicking the structure of phthalazine, and thus the anti-cancer potency of the core constituent can be conserved.

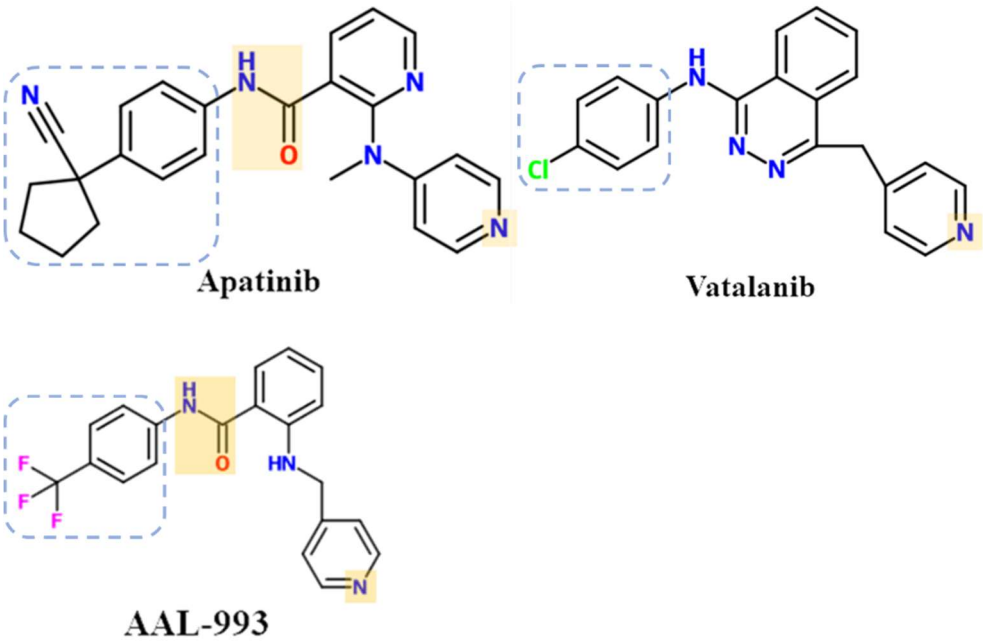

Figure 2. The chemical structures of apatinib, vatalanib, and AAL-993. These three compounds share structural similarities (orange) which confer the capability to interact with the target proteins, while they are comprised of a hydrophobic bulk with distinctive features (blue dotted).

The strong binding affinity of apatinib may be attributed at least in part to the nitrile group present in the bulky 4-cyclopentylphenyl ring constituent, though the detailed mechanism still needs to be elucidated. In drug-protein interaction, nitriles often act as hydrogen bond acceptors, and the nitrile nitrogen can interact with amino acid residues, such as serine or arginine as expected for hydrogen bond donors, or water molecules which in turn are immobilized in the binding site (Fleming, Yao, Ravikumar, Funk, & Shook, 2010). Besides, nitriles in most cases are not readily metabolized and are eliminated unchanged. Apatinib is an α-aryl acetonitrile-bearing compound that contains the nitrile group on a quaternary carbon adjacent to an aromatic ring. Positioning the nitrile on a fully substituted carbon prevents oxidation at the nitrile-bearing carbon and thereby prevents the release of toxic cyanide.

1.3.2. Mechanism of action

Apatinib is a multi-kinase inhibitor that possess potential antiangiogenic and antineoplastic activities. It selectively binds to and inhibits the kinase activity of VEGFR2 with a half maximal inhibitory concentration (IC_{50}) value of 1 nM (Tian et al., 2011). Apatinib inhibits VEGF-stimulated endothelial cell migration and proliferation and decrease tumor microvessel density. It also mildly targets c-Kit and c-SRC tyrosine kinases. These kinases play an important role in tumor angiogenesis and have been actively investigated as targets for development of novel drugs since decades. In comparison with other tyrosine kinase inhibitors preclinically and/or clinically investigated, apatinib has a VEGFR2-binding affinity incredibly higher than vatalanib (IC_{50} value = 37 nM) and AAL-993 (IC_{50} value = 23 nM), as well as sorafenib (IC_{50} value = 90 nM), regorafenib (IC_{50} value = 4.2 nM), and lenvatinib (IC_{50} value = 4 nM) in cell-free assay conditions (Manley et al., 2002; Matsui et al., 2008; Wilhelm et al., 2004; Wilhelm et al., 2011; Wood et al., 2000). As increased expression of VEGFR2 has been frequently observed among patients with various types of cancer including HCC, the high affinity of apatinib to VEGFR2 creates significant therapeutic potential for it as an anti-cancer drug through suppressing its expression and subsequently angiogenesis.

VEGFR2 is a type III transmembrane kinase receptor which is comprised of an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic tyrosine kinase domain (E. Li & Hristova, 2006). In the kinase domain, the highly conserved DFG motif (Asp-Phe-Gly) located at the start of the activation loop, positioning at the residues 1046-1048, is capable of sterically blocking the binding of ATP by changing its conformation, and is a structural determinant of VEGFR2

activation (Aziz et al., 2016). When the DFG motif is in an out conformation ("DFG-out"), the side chain of Phe-1047 residue orientates toward the ATP binding pocket, forming a new allosteric site to prevent the entry of ATP into the pocket, and consequently the kinase domain is in its inactive state. On the other hand, in the "DFG-in" conformation, the side chain of Phe-1047 residue moves out of the pocket, allowing for ATP binding, thus the kinase domain becomes activated.

Most VEGFR2 inhibitors target the tyrosine kinase domain. For example, lenvatinib selectively binds to the "DFG-in" conformation, thereby blocking the binding of ATP to the kinase, while many others involving apatinib, AAL-993, vatalanib, sunitinib, sorafenib, and regorafenib bind to the "DFG-out" conformation, locking the kinase in its inactive state (Elmeligie et al., 2019; Manley et al., 2004; McTigue et al., 2012; Okamoto et al., 2015; Y. Wang et al., 2019; Zhao et al., 2014). According to the difference in the kinase conformation binding selectivity, inhibitors targeting the "DFG-in" state are classified as Type I inhibitors, whereas those binding to the "DFG-out" state are commonly referred to as Type II inhibitors.

The chemical mechanism of how apatinib interacts with VEGFR2 is not clear. A structural analysis (López & Menendez, 2015) has proposed that in its analog AAL-993, the N atom positioned in the terminal pyridine ring interacts with the hydrogen atom at the N-terminus of Cys-919 residue in the VEGFR2. The O atom positioned in the peptide bond interacts with the hydrogen atom at the N-terminus of Asp-1046 residue and the NH group interacts with the carboxylic terminus at the Glu-885 residue in the receptor. In addition, the phenyl ring of the core anthranilamide is sandwiched between the hydrophobic side chains of Val-916 and Lys-868 residues. The bulky

phenyl substituent fits the lipophilic pocket of VEGFR2. As depicted in **Figure 3**, these findings may provide insight into how apatinib binds to the tyrosine kinase domain of the VEGFR2 receptor.

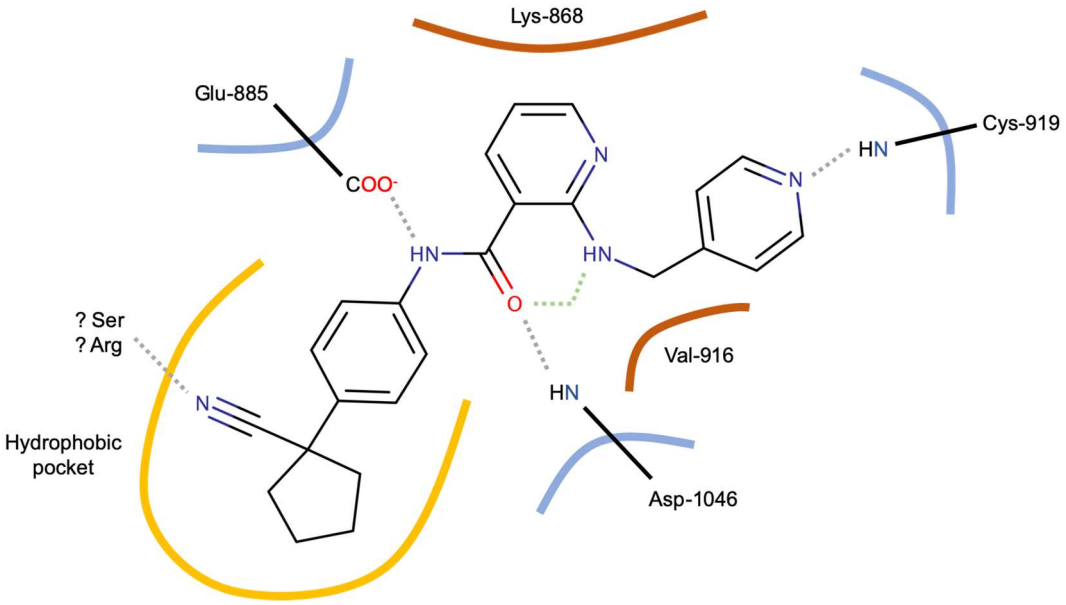

Figure 3. A hypothesized mechanism of apatinib binding to VEGFR2. Apatinib is stabilized itself by an intramolecular hydrogen bonding formed at the core anthranilamide (green dotted). Upon interaction, the core anthranilamide is sandwiched between the Val-916 and Lys-868 residues, whereby the core peptide bond is interacted via intermolecular hydrogen bonding by the Glu-885 and Asp-1046 residues (grey dotted). Additionally, the terminal pyridine ring is interacted via a hydrogen bond with the Cys-919 residue (grey dotted). These interactions interfere with the intramolecular hydrogen bonding and possibly cause a change in the three-dimensional structure of apatinib. The bulky phenyl substituent is stabilized within the hydrophobic pocket possibly via an interaction between the nitrile group and a serine or arginine residue.

1.3.3. Clinical trials

Apatinib is recently undergoing clinical trials on patients with various types of cancer. In 2009, the first clinical trial for apatinib was undergone on patients with chemotherapy-refractory advanced metastatic gastric cancer, with encouraging outcomes revealing that apatinib has an effective anti-tumor activity with controllable side effects (J. Li et al., 2010). Since then, clinical trials for apatinib have been spread to other types of solid tumor including HCC. In 2014, Qin et al. underwent a phase II, multicenter, randomized, open-label study for apatinib, enrolling 121 Chinese patients with advanced HCC. The study recommended the dose for apatinib monotherapy as high as 750-850 mg/day. The median time-to-progression (mTTP) and median overall survival (mOS) were 3.3-4.2 and 9.7-9.8 months, with a disease control rate (DCR) of 43.8%. The recommended doses, however, were not tolerated by many HCC patients especially with cirrhotic liver diseases as reported in late studies, those of which reduced the dose to 500 mg/day or below. In 2017, a phase I, single-center, open-label study (Kong et al., 2017), enrolling 22 Chinese patients with advanced HCC, documented that apatinib at the dose of 250 mg/day showed an objective response rate (ORR) and DCR of 40.9% and 81.8%, with a mTTP reaching 10.4 months.

Table 1. Clinical trials of apatinib on patients with advanced HCC. ORR, objective response rate; DCR, disease control rate; mTTP, median time-to-progression; mPFS, median progression-free survival; mOS, median overall survival.

Study	Trial	No. of patients enrolled	Ethnicity	Treatment	Dose	ORR (%)	DCR (%)	mTTP (month)	mPFS (month)	mOS (month)
Qin et al., 2014	Phase II, multicenter, randomized, open-label	121	Chinese	Apatinib	750-850 mg/day	5	43.8	3.3-4.2	N/A	9.7-9.8
Kong et al., 2017	Phase I, single-center, open-label	22	Chinese	Apatinib	250 mg/day	40.9	81.8	10.4	N/A	N/A
Lu et al., 2017	Phase I, single-center, randomized controlled	44	Chinese	Apatinib plus TACE vs TACE alone	500 mg/day	35	60	N/A	12.5	N/A
Chen et al., 2018	Phase I, single-center, randomized controlled	80	Chinese	Apatinib plus TACE vs TACE alone	500 mg/day	45.8	58.3	6.3	N/A	13
Yu et al., 2018	Phase I, single-center	31	Chinese	Apatinib	500 mg/day	32.26	80.65	4.8	N/A	N/A
Xu et al., 2019	Phase I, single-center, open-label	18	Chinese	Apatinib plus camrelizumab	125-250 mg/day	50	93.8	N/A	5.8	N/A
Yang et al., 2019	Phase I, single-center, randomized controlled	47	Chinese	Apatinib plus TACE vs TACE alone	250 mg/day	36	56	N/A	4.5	16.5
Li et al., 2020	Phase III, multicenter, randomized, placebo controlled, double-blind	393	Chinese	Apatinib	750 mg/day	10.7	N/A	N/A	4.5	8.7

In 2017, a phase I, single-center, randomized controlled study was carried out to evaluate the combination effect of apatinib and transcatheter arterial chemoembolization (TACE) on 44 Chinese HCC patients, showing an improved median progression-free survival (mPFS) as long as 12.5 months (Lu et al., 2017). A later study reported similar results in a retrospective study, with higher response rate observed in the TACE-apatinib combination group (Chen, Yu, Zhang, & Liu, 2018). A more recent publication also reported that TACE-apatinib combination treatment significantly prolonged the mPFS and mOS in patients with advanced HCC compared with TACE treatment alone (Z. Yang et al., 2019). In 2018, a phase I, single-center, open-label study, enrolling 18 Chinese patients, was underwent to evaluate the combination of apatinib with camrelizumab, an anti-PD-1 humanized monoclonal antibody, on advanced HCC. The ORR and DCR were 50% and 93.8% respectively, with a mPFS of 5.8 months (J. Xu et al., 2019). Although most of the studies are performed in single-center with a small sample size, these studies provide evidence that apatinib has promising potential for developing combination treatment with current therapeutic practices and agents to enhance the therapeutic effects on advanced HCC in the near future. In addition, the recently disclosed results from a phase III, multicenter, randomized, placebo controlled, double-blind trial (Q. Li et al., 2020) for assessing the efficacy of apatinib on 393 Chinese patients with advanced HCC proved that apatinib significantly prolonged the mOS (8.7 months vs 6.8 months) and mPFS (4.5 months vs 1.9 months) of the patients in apatinib-receiving group compared to those with placebo, with manageable tolerance.

Many clinical studies have reported that apatinib was well tolerated by the patients, though the occurrence of adverse effects was positively proportional to the

dose used. A considerable number of patients indeed showed intolerance to higher doses, and they required to reduce the dose for a period of time or to terminate the drug treatment. The safety and application of apatinib in patients seems to be strictly limited by the concentration effect. For future drug development, it is essential to develop feasible combination therapy regimes to enhance the therapeutic potential of apatinib in HCC.

CHAPTER 2

MATERIALS AND METHODS

Section 2.1 Materials

2.1.1. Agents

Apatinib was purchased from MedChemExpress (New Jersey, US). For all *in vitro* assays, apatinib was first dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at 100 mM as the stock concentration before further diluted with culture medium to the desired concentrations.

2.1.2. Cell lines

Cell line	Characteristics	Source
PLC/PRF/5	HCC	Japanese Cancer Research Bank (JCRB0406)
MHCC-97L	Metastatic HCC	Liver Cancer Institute, Fudan University
PLC/PRF/5-AR	Apatinib-resistant clone	Derived from PLC/PRF/5 in laboratory
MHCC-97L-AR	Apatinib-resistant clone	Derived from MHCC-97L in laboratory
293FT	Human embryonal kidney cells transformed with the SV40 large T antigen	Invitrogen

2.1.3. Reagents for cell culture

Reagent	Vendor
Subculture	
Gibco™ Dulbecco's Modified Eagle Medium (DMEM) with High Glucose	Thermo Fisher Scientific
Gibco™ Fetal Bovine Serum (FBS)	Thermo Fisher Scientific
Gibco™ Penicillin-Streptomycin	Thermo Fisher Scientific
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Trypan Blue	Invitrogen
Migration and Invasion assay	
Matrigel™ Matrix	Corning (Corning, NY)
shRNA-based gene knockdown	
Polybrene	Sigma-Aldrich
Lipofectamine™ 2000 transfection reagent	Invitrogen
Puromycin	Sigma-Aldrich

2.1.4. Other reagents for experimental use

Reagent	Vendor
Cell proliferation assay	
MTT	
Flow cytometry	
Annexin V-FITC reagent	BioVision (Milpitas, CA)
10X Annexin V binding buffer	BD Biosciences
SDS-PAGE and Western blotting	
RIPA lysis buffer	Cell Signaling Technology
NETN lysis buffer	Prepared in laboratory
PhosSTOP™	Roche (Basel, Switzerland)
cOmplete™ EDTA-free protease inhibitor cocktail	Roche
6X Laemmli buffer	
1X SDS-PAGE running buffer	Prepared in laboratory
Methanol	
1X Western blotting transfer buffer	Prepared in laboratory
5% skimmed milk	Prepared in laboratory
1X TBST washing buffer	Prepared in laboratory
WesternBright™ ECL kit	Advansta
WesternBright™ Quantum kit	Advansta
Migration and invasion assay	
Matrigel™ Matrix	Corning
Crystal violet	Sigma-Aldrich
RNA isolation	
TRIZOL Reagent	Invitrogen
Chloroform	
Isopropanol	
75% ethanol	Prepared in laboratory
UltraPure™ DNase/RNase-free distilled water	Invitrogen
Real-time PCR (qPCR)	
PrimeScript™ RT reagent Kit	TaKaRa
BrightGreen 2X qPCR MasterMix-ROX	Abm (Vancouver, Canada)

2.1.5. Primer sequences for qPCR analysis

Gene symbol (GenBank Number)	Forward primer (5'-3')	Reverse primer (3'-5')
CYP1A1 (NM_000499.5)	TTTGGAGCTGGGTTTGACAC	TTACAAAGACACAACGCCCC
GAPDH NM_001256799.3	CCTCCTGGCGTCGTGATTAGTG	CAGAGGGCTACAATGTGATGG

2.1.6. Short-hairpin RNA (shRNA) sequences

Gene symbol (sequence number)	Nucleotide sequence (5'-3')
CYP1A1 (20)	CCGGCGACAAGGTGTTAAGTGAGAACTCGAGTTCT CACTTAACACCTTGTCGTTTTTG
CYP1A1 (21)	CCGGCTGTCTGGTATTCTGGGTAATCTCGAGATTAC CCAGAATACCAGACAGTTTTTG
Non-target control	CCGGTTGTGCTCTTCATCTTGTTGCCGGCAACAAGA TGAAGAGCACCAATTTTTG

2.1.7. Antibodies

Antibody	Characteristics	Vendor	Usage
β-Actin	Mouse monoclonal	Sigma-Aldrich	Western blotting
α-Tubulin	Mouse monoclonal	Sigma-Aldrich	Western blotting
Bax	Rabbit monoclonal	Cell Signaling Technology	Western blotting
Bcl-2	Rabbit monoclonal Mouse monoclonal	Cell Signaling Technology	Western blotting
CYP1A1	Rabbit polyclonal	Origene	Western blotting

Section 2.2 Methods

2.2.1. Cell culture

Two human HCC cell lines, PLC/PRF/5 and MHCC-97L were used in the present study. The cancer cells were maintained in Dulbecco's modified eagle medium with high glucose (DMEM/HG; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific). PLC/PRF/5 and MHCC-97L were employed to establish apatinib-resistant HCC cell lines named PLC/PRF/5-AR and MHCC-97L-AR respectively. The resistant cells were established by exposure to increasing concentrations of apatinib from an initial concentration of 5 μM to 20 μM as the final concentration for 6 months. Then, the established cell lines were maintained in the medium supplemented with apatinib at the final concentration. The mock cell lines were maintained in the medium supplemented with DMSO at equivalent

concentration. All cells were cultured at 37°C in a humid atmosphere (5% CO2, 95% air).

2.2.2. Cell proliferation assay

PLC/PRF/5 cells (1.5×10^3 per well) and MHCC-97L cells (2×10^3 per well) seeded in 96-well plates were treated with apatinib at different concentrations for 72 hours. Then, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by replacing the medium with new culture medium containing 10% of MTT (5 mg/mL) for 3 hours. Light absorbance was measured at 570 nm on a microplate reader (BMG Labtech, Germany).

2.2.3. Cell apoptosis assay

Cell apoptosis was detected by Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining followed by flow cytometry (Accuri^{TM} C6 flow cytometer, BD Biosciences, New Jersey, USA). Cells (3×10^4 per well) seeded in 12-well plates were exposed to apatinib at different concentrations for 48 hours. Then, the cells were trypsinized and stained with Annexin V-FITC and PI for 10 minutes. The apoptotic cells were quantified by measuring the percentage of Annexin V-FITC-positive cells.

2.2.4. Western blotting

Cell lysates were prepared using radioimmunoprecipitation assay (RIPA) lysis buffer (Cell Signaling Technology) or NETN lysis buffer containing cocktail protease inhibitor and phosphatase inhibitor (Roche). Protein samples were mixed with 6X Laemmli buffer and denatured at 100°C for 5 min, separated by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). After blocking with 5% skimmed milk for 30 minutes, the membranes were incubated with primary antibodies against Bax (1:1000; Abcam), Bcl-2 (1:1000; Abcam), CYP1A1 (1:1000; Origene), α-tubulin (1:1000; Sigma-Aldrich), and β-actin (1:1000; Sigma-Aldrich) overnight at 4 °C. After washing in Tris-buffered saline with Tween-20 (TBST) buffer for 1 hour, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature and further washed with TBST buffer for 2 hours. The signal was detected using WesternBright™ enhanced chemiluminescence (ECL) kit or WesternBright™ Quantum kit (Advansta).

2.2.5. Cell migration and invasion assay

Transwell cell migration assays were performed using BD Falcon Cell Culture Inserts. 5×10^4 cells were seeded in the inserts containing serum-free medium. The outer chambers were filled with 1 ml of medium containing 10% FBS (PLC/PRF/5 and PLC/PRF/5-AR) or conditioned medium (MHCC-97L and MHCC-97L-AR). The cells were allowed to migrate for 24 hours. After incubation, non-migrating cells remaining on the upper surface of the insert were removed with cotton swab. Migrated cells were fixed by using 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 minutes and stained with crystal violet for 1 hour at room temperature. The migrating cells were observed under a light microscope and counted by using ImageJ software.

In the invasion assay, 1×10^5 cells (PLC/PRF/5 and PLC/PRF/5-AR) and 5×10^4 cells (MHCC-97L and MHCC-97L-AR) were seeded in the inserts coated with

Matrigel and allowed to invade for 24 hours. Invading cells were stained and analyzed as described above.

2.2.6. RNA sequencing and data analysis

Total RNA from mock and apatinib-resistant HCC cells derived from PLC/PRF/5 and MHCC-97L as well as the knockdown clone shCYP1A1#20 derived from each mock control were extracted as described. RNA quality was confirmed with Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA) to have an OD260/280 ratio within the range of 1.8 to 2.0 and an RNA integrity number (RIN) value higher than 8.0. RNA-sequencing was performed with Illumina Solexa sequencing using Hiseq 1500 sequencer (Illumina, San Diego, CA). Indexed library construction, HiSeq sequencing run (pair-end sequencing of 101bp). The primary analysis was performed on the transcriptome data. The transcriptome data generated from RNA sequencing were imported into Microsoft Excel software for calculation of gene expression in the apatinib-resistant HCC cells.

Genes detected with Transcripts per Million (TPM)<1 were considered as undetectable and excluded from the counted gene lists. The counted genes were ranked according to the fold change in gene expression calculated by the ratio of TPM in apatinib-resistant cells over TPM in mock control cells. Ratios greater than 1 were regarded as up-regulated while ratios smaller than 1 were regarded as down-regulated. Subsequently, the top-ranked 30 up-regulated genes in each apatinib-resistant HCC cells were listed in terms of descending TPM ratio. A common target candidate with the highest gene upregulation was selected from the ranked lists.

The secondary analysis was performed using the Gene Set Enrichment Analysis (GSEA) or the Ingenuity Pathway Analysis (IPA) software. The gene expression data were employed in the GSEA to investigate the overall alternation in metabolic signaling pathways, and to reveal the associations between the common target and the pathways in the apatinib-resistant cells and the lentiviral-based knockdown cells. The IPA was used to explore the possible signaling pathways associated with the gene knockdown in HCC cells. Down-regulation or up-regulation of a signaling pathway was determined by its activation Z-score which was regarded as negative or positive.

2.2.7. RNA isolation, sequencing analysis and qRT-PCR

Total RNA was extracted using RNAiso Plus (TaKaRa, Japan) according to the manufacturer's protocol. Total RNA samples were sent to Queen Elizabeth Hospital, Kowloon, Hong Kong for sequencing. The sequencing results obtained were subsequently compiled into gene set enrichment analysis (GSEA) for pathway analysis. The extracted RNA was quantified using NanoDrop^{TM} One/One^{C} Microvolume UV-Vis Spectrophotometer (Thermo Scientific, USA). Then, complementary single strand DNA (cDNA) was synthesized using PrimeScript^{TM} RT Master Mix (TaKaRa, Dalian, China). For the quantitative PCR, BrightGreen 2x qPCR MasterMix-ROX (Coderegenesis Biosciences) was used. The PCR primers were designed by using National Center for Biotechnology Information (NCBI) Primer-BLAST as described in **Materials**. Experiments were independently performed in triplicate. Data are shown as mean ± standard deviation (SD).

2.2.8. Construction of human CYP1A1 shRNA

The human CYP1A1 shRNA was designed by taking reference from the SIGMA mission website. The shRNA sequences were designed as described in **Materials**. The shRNA was re-annealed and cloned by using PCR method, ligated by T4 DNA ligase (Invitrogen) with the plasmid vector pLK0.1-Puro.

2.2.9. DNA gel electrophoresis and plasmid transformation

The ligated product was confirmed by DNA gel electrophoresis, and then transformed into E. coli DH5α cells by using heat shock method at 42°C for 1.5 minutes. The transformed cells were subsequently cultured in Lysogeny broth without puromycin for 30 minutes and spread on agar plates with adding puromycin for colony selection. The plates were incubated at 37°C overnight.

2.2.10. Lentiviral-based gene knockdown

shCYP1A1 knockdown cells were established through lentiviral-based method. 293FT cells were seeded in 60mm culture dish and transfected with packaging mix and lentiviral vectors containing the shRNA sequences when 90% cell confluency was reached. The transfection medium was replaced by fresh culture medium supplemented with 20% FBS after 24 hours. The lentivirus-containing medium was collected 48 hours after the transfection and was centrifuged at 3,000 rpm for 5 minutes at 4ºC prior to infection. HCC cells were infected by directly adding the lentivirus-containing medium to the culture medium of HCC cells together with polybrene (Sigma) at final concentration of 8 μg/mL. The infected HCC cells were selected by 1 to 2 μg/mL of puromycin (Sigma) depending on cell lines. The knockdown efficiency was confirmed by Western Blot analysis and qPCR analysis.

2.2.11. CYP1A1 enzymatic activity assay

The enzymatic activity of CYP1A1 was measured by using the Promega P450-Glo™ CYP1A1 Assay System V8751. Mock and apatinib-resistant cells derived from PLC/PRF/5 were seeded with a density of 2,000 cells/well in 96-well plates. After 24 hours, the cells were washed with PBS, and the culture medium was replaced with fresh medium containing the luminogenic substrate according to the manufacturer's protocol. Fine adjustments were made: Luciferin-CEE final concentration with 1:100 dilution, and medium volume 40μl/well. Following incubation for three hours at 37°C, 25μL of culture medium was transferred from each well to a 96-well opaque white luminometer plate at room temperature. Then, 25μL of luciferin detection reagent was added to the wells, followed by further incubation for 20 minutes at room temperature. The luminescence was detected by CLARIOstar reader (BMG Labtech, Germany).

2.2.12. Statistical analysis

All statistical analyses were performed using the statistical software including IBM SPSS Statistics 21 (SPSS Inc., Chicago, USA) and GraphPad Prism 6 (GraphPad Software Inc., California, USA). Two-tailed $P < 0.05$ was considered statistically significant.

CHAPTER 3

ESTABLISHMENT AND CHARACTERIZATION OF APATINIB RESISTANT HCC CELLS

Section 3.1 Introduction

HCC accounts for more than 70% of liver cancer cases worldwide and is highly malignant, recurrent, and drug-resistant. It is usually diagnosed at advanced stages and thus inoperable. Although sorafenib has long been prescribed as the standard first-line drug treatment for advanced HCC over more than a decade, the treatment outcome is often ineffective and post-therapy recurrence rate is frequently high. The inefficacy of sorafenib treatment can be attributed to the fact that cancer cells develop acquired drug resistance during treatment of tumors that were initially sensitive. In addition, acquired resistance can be caused by mutations arising during treatment and through various other adaptive responses mediated by resistance drivers or by the tumor microenvironment **(Figure 4)**.

The lack of therapeutic options for HCC stimulates the emergence of new classes of cancer therapeutics, such as orphan drugs. Apatinib is an orphan drug candidate that has been approved since 2014 in China for advanced gastric cancer, and is increasingly receiving awareness on its potential effectiveness against other types of solid tumor including advanced HCC. Multiple clinical trials have been promptly carried out to evaluate the safety and efficacy of apatinib on HCC patients and are receiving encouraging results. However, current understanding of acquired resistance to apatinib in cancer is very limited. Only a handful of studies have revealed some key players that mediate acquired resistance to apatinib in advanced gastric cancer, and the knowledge about such resistance in other types of cancer like HCC remains largely unknown.

Previous studies have focused on acquired drug resistance as a major hindrance to chemotherapy and molecularly targeted therapy for HCC. Although various mechanisms of acquired chemoresistance have been identified using *in vitro* and *in vivo* models, these mechanisms do not solely mediate the resistance and do cooperate with each other or multiple pathways through protein-protein interactions, many of them involving dysregulated metabolic signalling pathways. The cross-linking of these mechanisms largely limits the applicability of single drugs in HCC treatment. In addition, based on the fact that most of current drugs widely used in cancer therapy are commonly kinase inhibitors that target different ranges or groups of kinase, cancer cells may develop different ranges of acquired resistance mechanism corresponding to the drug actions. Since clinical trials studying apatinib on HCC have been started in China for years and will be conducted on a geographically larger scale in the near future, apatinib is receiving interest from researchers worldwide, with more literature available on its potential for cancer treatments in combination with other therapeutic agents. It is of utmost urgency to study the molecular mechanisms underpinning the acquired resistance to apatinib in HCC in order to increase the applicability of apatinib in cancer therapy. Hence, the foremost objective of this study is to identify a key player that regulate the mechanisms of acquired resistance to apatinib.

To attain this objective, we established apatinib-resistant human HCC cell lines derived from PLC/PRF/5 and MHCC-97L cell to investigate the potential pathways mediating the resistance. Human HCC cells were continuously exposed to increasing concentrations of apatinib until reaching a final concentration at which the cells were maintained. In response to the drug treatment, the cell growth decreased whereas the cell death increased, presumably as apatinib inhibited the cell growth and killed most

of the sensitive HCC cells. After an extensive time period of treatment with apatinib for 6 months, most of the cells that survived were believed to be resistant to apatinib treatment **(Figure 5)**. *In vitro* functional assays including apoptosis assay, Western blot, and migration/invasion assay, were performed to characterize and confirm the apatinib-resistant HCC cell lines.

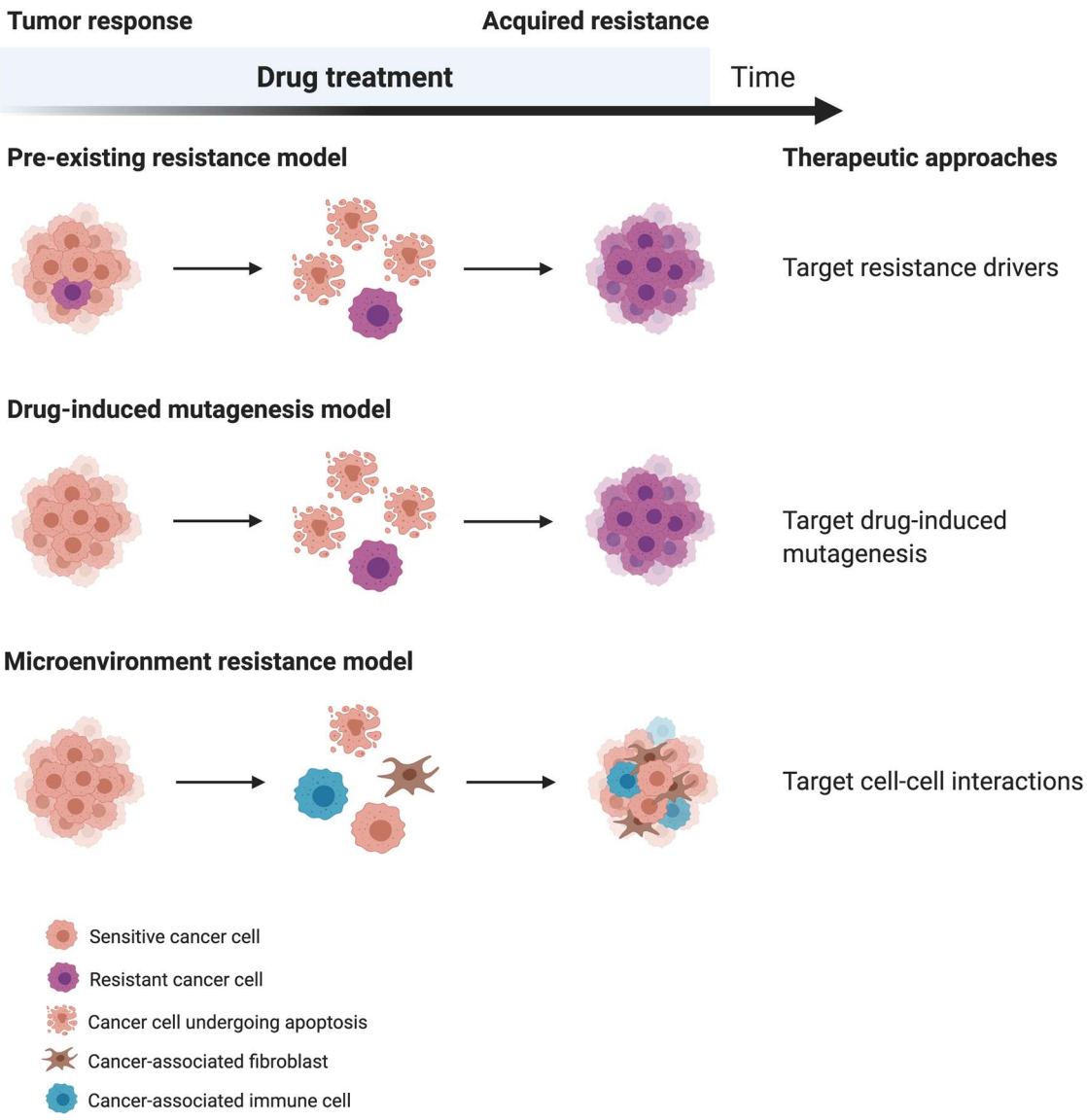

Figure 4. Models of acquired drug resistance in cancer cells. Cancer cells develop acquired drug resistance during treatment of tumors that initially respond to the drug. Resistant cancer cells survive after various rounds of drug treatment. Current models of acquired drug resistance mechanism in cancer include pre-existing resistance, drug-induced metagenesis, and tumor microenvironment-mediated resistance. Each provides distinct therapeutic opportunities, but multiple mechanisms may occur simultaneously within the tumor.

Section 3.2 Experimental Scheme

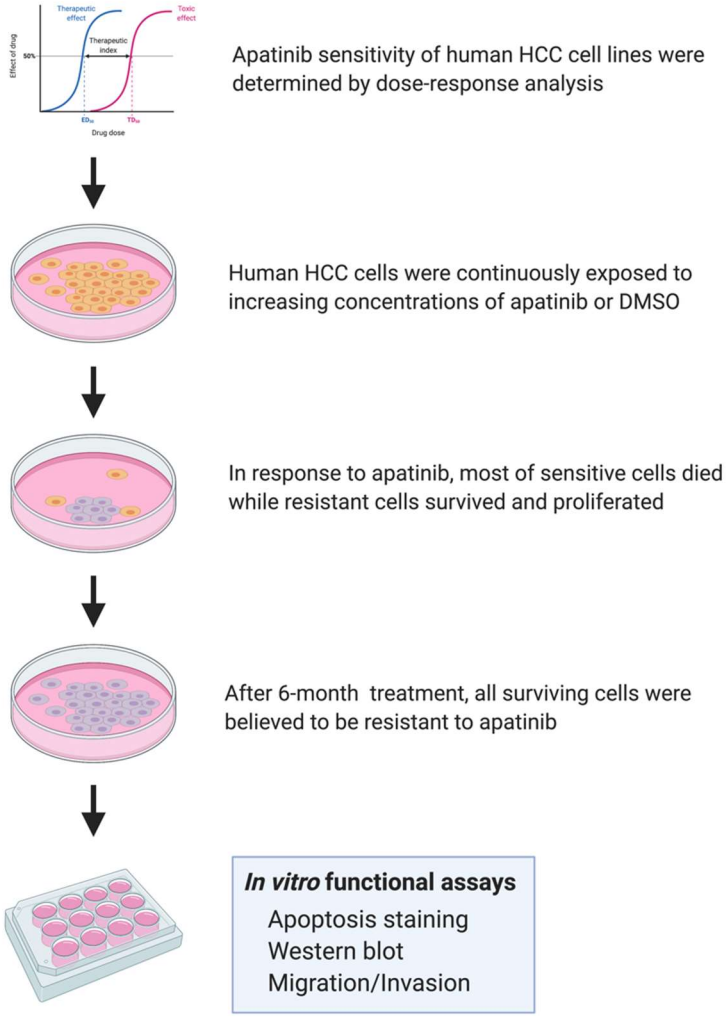

Figure 5. Illustrated experimental scheme for establishing and characterizing apatinib-resistant HCC cell lines.

Section 3.3 Results

3.3.1. Human HCC cell lines showed differential sensitivity to apatinib

In order to investigate the mechanism of apatinib resistance in HCC *in vitro*, apatinib-resistant HCC cell models were established. Apatinib sensitivity of human HCC cell lines were tested by dose-response analysis as a pilot study to determine the initial dose for establishing the drug-resistant cell models. Apatinib sensitivity was examined in two human HCC cell lines, PLC/PRF/5 and MHCC-97L using MTT assay (**Figure 6**). The cells were exposed to different concentrations of apatinib for 72 hours. Higher inhibition effect on cell growth was detected in MHCC-97L (IC_{50} value = 17.65 μM) compared with PLC/PRF/5 (IC_{50} value = 34.12 μM), indicating that MHCC-97L cells had a higher apatinib sensitivity than PLC/PRF/5 cells.

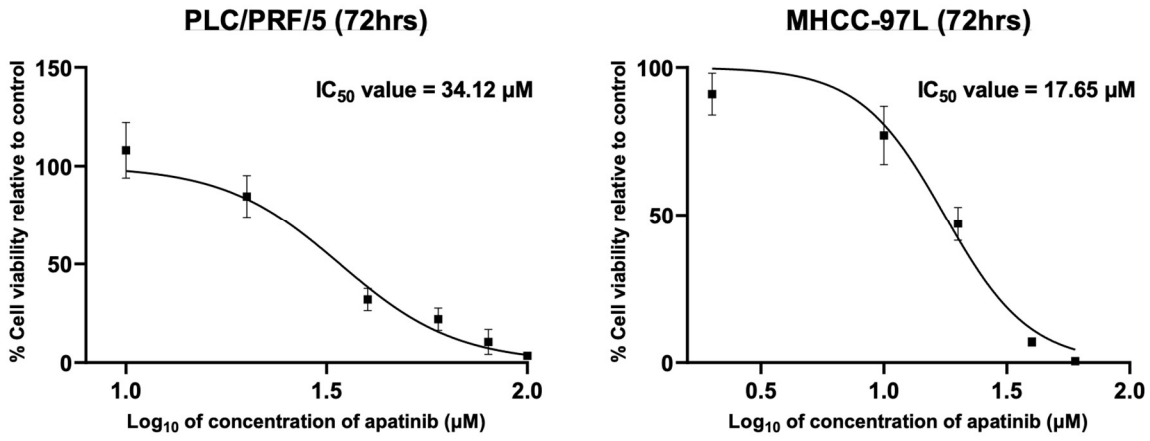

Figure 6. Human HCC cell lines had different sensitivity to apatinib with different IC_{50} values. The experiments were performed in triplicate. All data are shown as mean ± SD. The curves represent the best-fit curve for each cell line. The calculated IC50 value for each cell line is shown in the graph.

3.3.2. Establishment of apatinib-resistant HCC cell lines

Two human HCC cell lines, PLC/PRF/5 and MHCC-97L, were employed to establish apatinib-resistant cell models, namely PLC/PRF/5-AR and MHCC-97L-AR, in 6 months. The cells were continuously exposed to increasing concentrations of apatinib **(Figure 7)**. The HCC cells were initially treated with apatinib at 5 μM. In parallel, the mock cells were treated with DMSO at the corresponding dose as the control. During the first month, the dose was gradually escalated to 15 μM as the lower doses did not obviously suppress the cell growth. At 10 μM, the cell growth was marginally inhibited in MHCC-97L but not in PLC/PRF/5. When the dose was increased to 15 μM, both cell lines showed retarded cell growth, where significant retardation in the cell growth and proliferation was observed in MHCC-97L. The cells were maintained at 15 μM of apatinib for 5 months to allow the cells to recover and stabilize at the concentration, and later the dose was further escalated to 20 μM. Finally, the cells were maintained at 20 μM as the final concentration for a month before performing the functional assays.

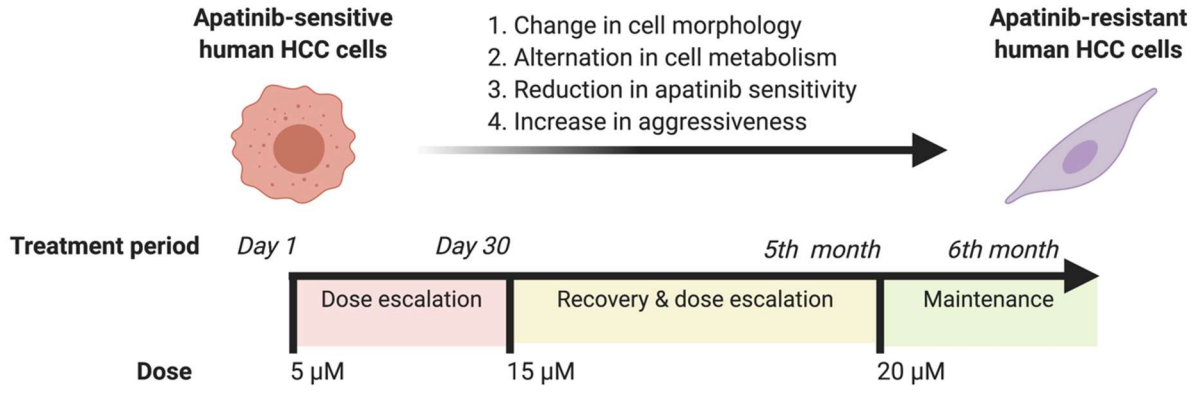

Figure 7. Establishment of apatinib-resistant human HCC cell lines in 6 months.

3.3.3. Apatinib-resistant HCC cells exhibited distinct morphological changes as compared to their mock controls

The morphology of apatinib-resistant HCC cells was observed under a bright-view microscope regularly during and after the establishment process. The resistant cells were morphologically distinct from their mock cells, having less cell-cell adhesion with fibroblast-like spindle-shaped morphology (**Figure 8**). In addition, the growth of the resistant cells was slower than the mock cells after the extensive establishment.

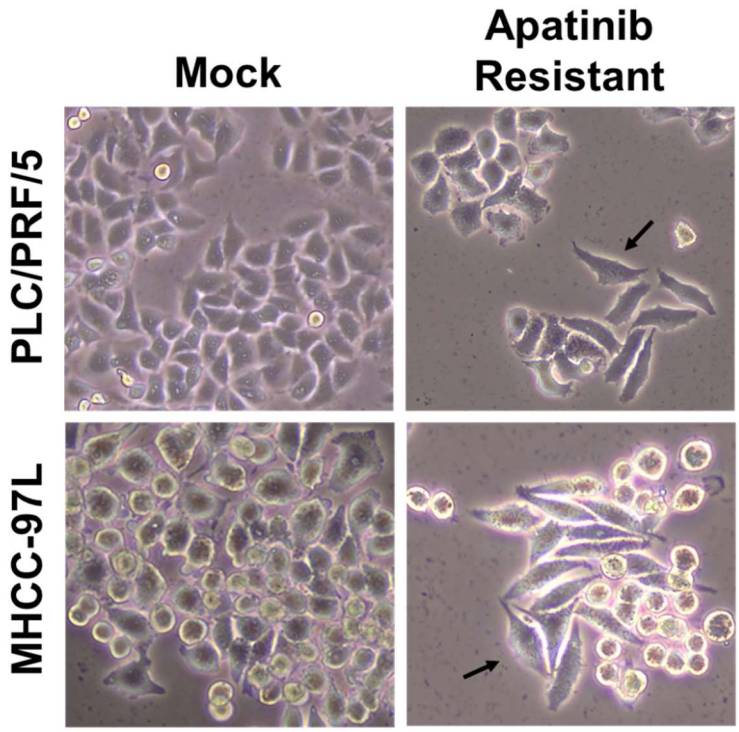

Figure 8. Apatinib-resistant HCC cells displayed distinctive morphological changes as compared to their mock cells. The black arrow indicates the resistant cells.

3.3.4. Apatinib-resistant HCC cells showed resistance to apatinib-induced apoptosis

The responsiveness of the HCC cells to apatinib-induced apoptosis was examined by Annexin V/PI staining. Compared with their mock control cells, PLC/PRF/5-AR cells grew more slowly when treated at 20 μM of apatinib in complete medium, and showed less apoptosis when being exposed to higher concentrations in the medium containing 2% FBS for 48 hours (12.79% vs 14.73%; 14.48% vs 24.98%; 18.77% vs 34.75% when treated with 30, 50 and 80 μM respectively). Similarly, compared with the mock, MHCC-97L-AR cells proliferated more slowly when maintained at 20 μM of apatinib in complete medium, and displayed less apoptosis when being exposed to 20 and 30 μM in the medium containing 5% FBS for 48 hours (9.56% vs 25.5%; 26.65% vs 41.8% respectively) (**Figure 9**). PLC/PRF/5-AR and MHCC-97L-AR cells were much resistant to apatinib.

Protein expression levels of apoptotic markers were examined by Western blot analysis (**Figure 10**). Apatinib-resistant cells displayed higher expression of endogenous Bcl-2 in both PLC/PRF/5 and MHCC-97L as compared with their mock control cells, when treated with apatinib at 50 and 80 μM for 48 hours. Bcl-2 functions as an anti-apoptotic regulator which suppresses the formation of pore on the outer mitochondrial membrane by dimerization of pro-apoptotic proteins like Bax to release cytochrome c inducing subsequent caspase cascade. Intriguingly, the expression levels of endogenous Bax did not show a significant difference in PLC/PRF/5-AR but were slightly increased in MHCC-97L-AR.

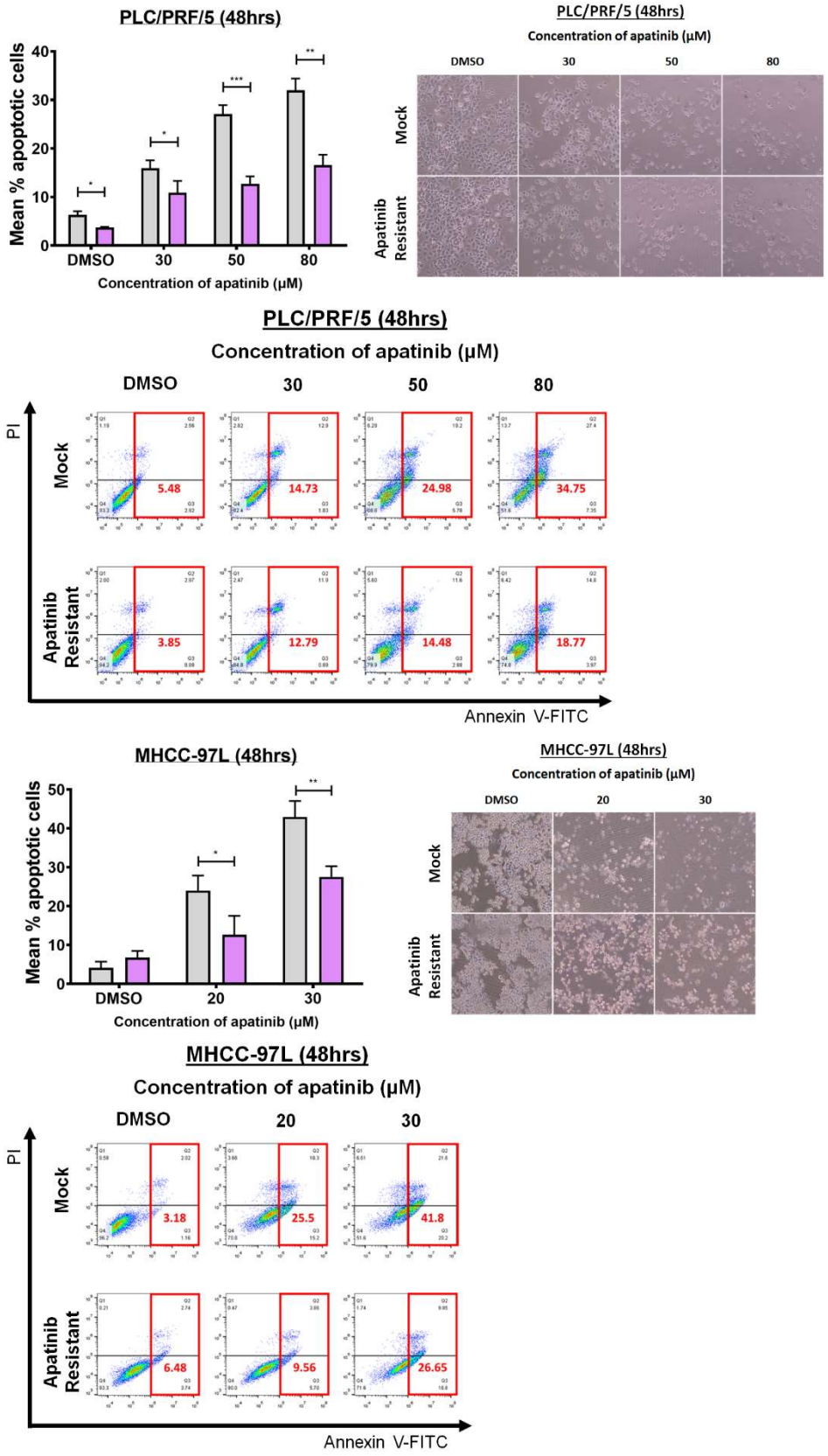

Figure 9. Apatinib-resistant HCC cells showed higher resistance to apatinib-induced apoptosis. The experiments were performed in triplicate. Student's t-test, *p<0.05; **p<0.01; ***p<0.001.

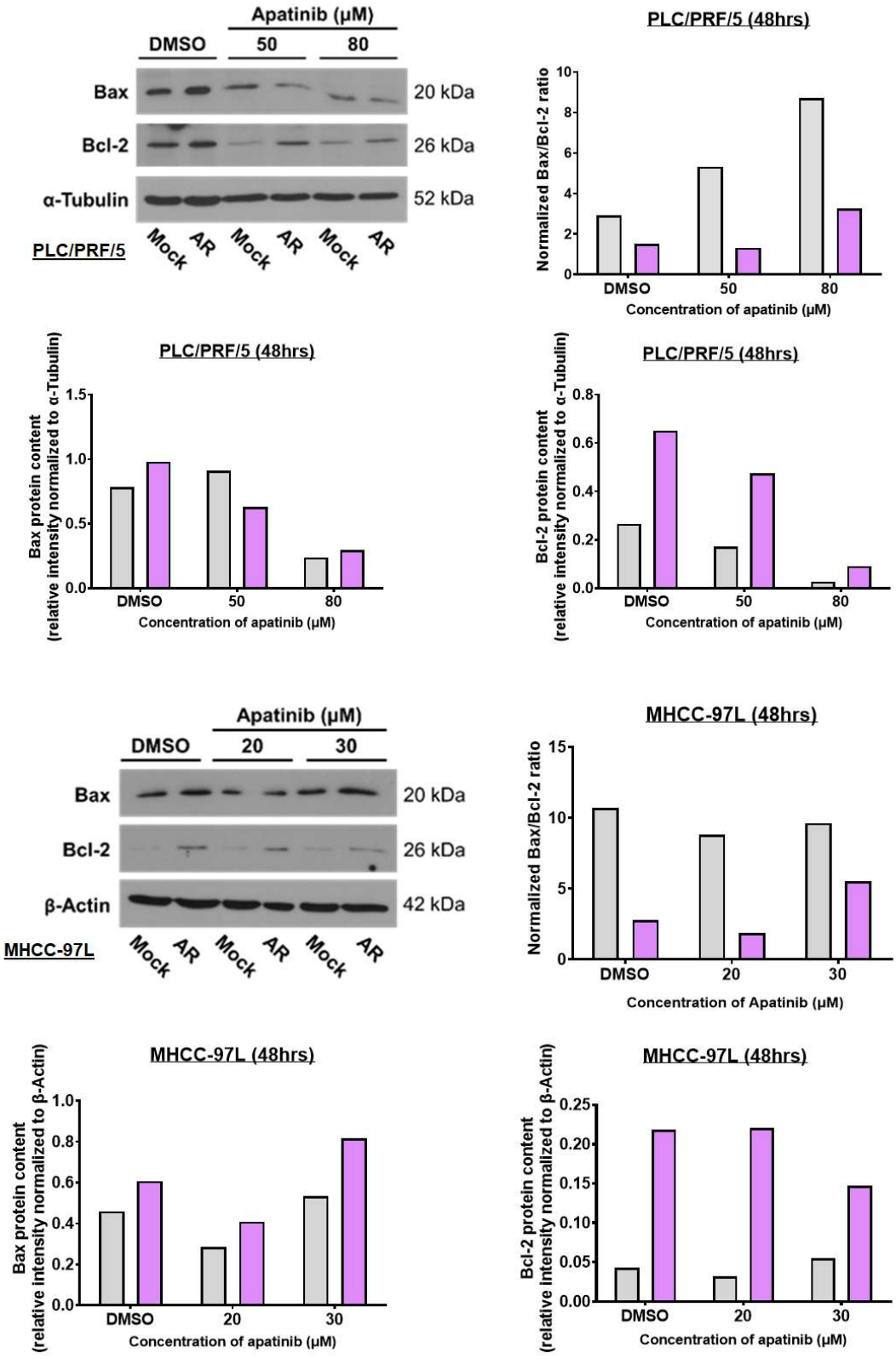

Figure 10. Apatinib-resistant HCC cells exhibited a lower ratio of Bax/Bcl-2 expression compared with their mock cells in response to apatinib treatment. AR, apatinib-resistant cells.

3.3.5. Apatinib-resistant HCC cells showed increased migration and invasion abilities

The migrating and invading potentials of apatinib-resistant human HCC cells were assessed using trans-well assays for 24 hours (**Figure 11**). In PLC/PRF/5, the migration capability of the resistant cells was increased by approximately 1.5-fold ($p < 0.01$), whereas the invasion capability of the cells increased by approximately 2-fold ($p < 0.01$), compared with its mock cells. In MHCC-97L, the migration capability of the resistant cells was increased by approximately 1.5-fold ($p < 0.05$), whereas the invasion capability of the cells was increased by approximately 1.6-fold ($p < 0.01$), compared with its mock cells. These results indicate that the resistant cells possessed higher potential to migrate and invade.

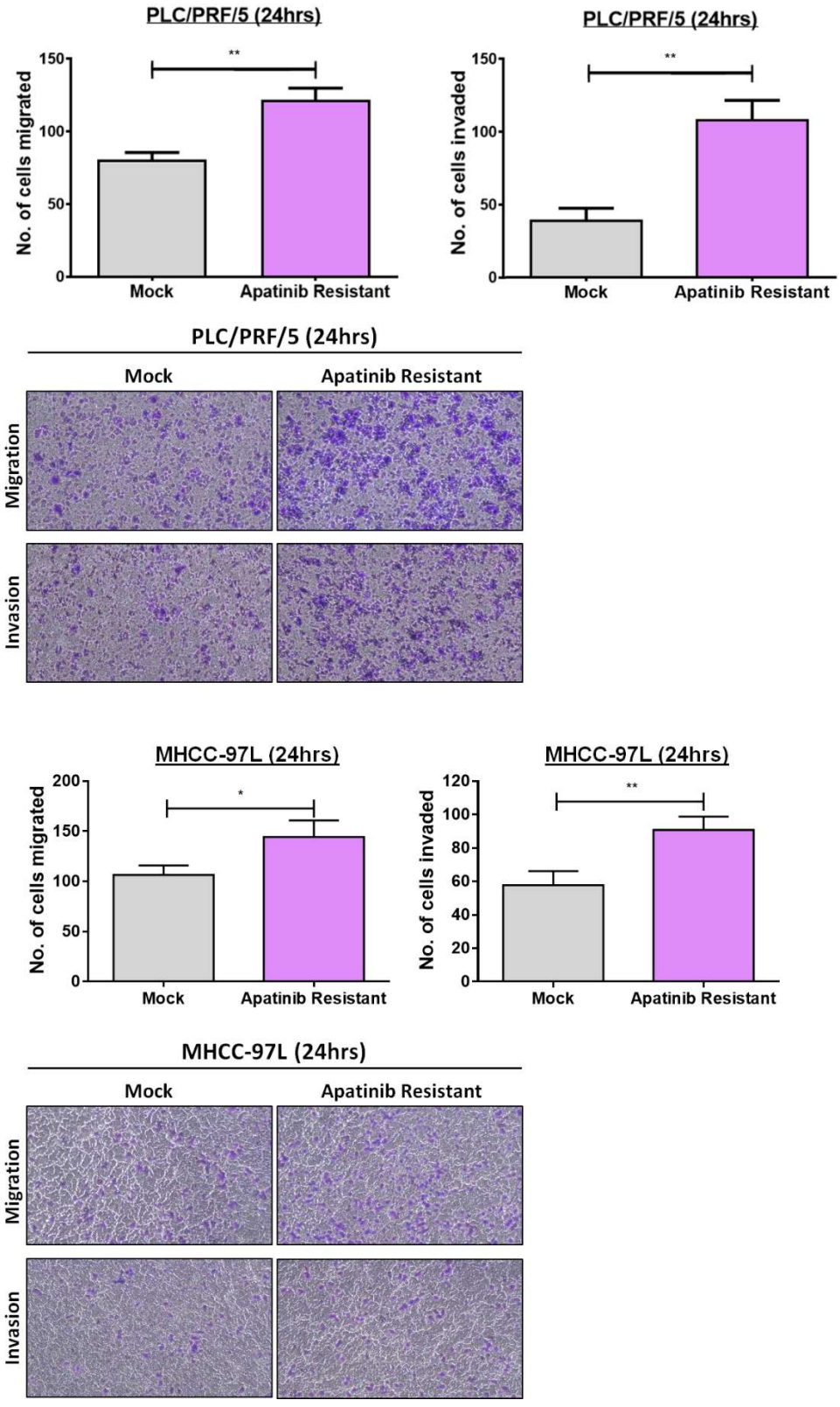

Figure 11. Apatinib-resistant HCC cells displayed higher migratory and invasive capabilities compared with mock cells. Each experiment was performed in triplicate. Student's t-test, *$p<0.05$; **$p<0.01$.

Section 3.4 Discussion

In this part of the study, two apatinib-resistant HCC cell models using human HCC cell lines PLC/PRF/5 and MHCC-97L were established by continuous exposure to escalating concentration of apatinib. There is limited therapeutic options for patients with advanced HCC. Apatinib has been recently proposed as a promising agent against HCC and is undergoing clinical trials in China. An increasing body of encouraging data has been reported. However, the problem of acquired drug resistance in cancer cells is inevitable. The current knowledge about apatinib resistance in HCC is lacking. The most direct and simplest way to study drug resistance in cancer cells is to establish a drug-resistant cell model. Clinically relevant models are developed with the aim of mimicking the conditions cancer patients experience during the treatment. The cell models were initially treated with lower doses of apatinib. The cells were sub-cultured in drug-free media and treated with apatinib the following day. When the cells became stable, the dose was gradually increased until the final concentration was reached. This strategy mimics the cycles of treatment a cancer patient receives in the clinic.

3.4.1. Apatinib-resistant HCC cells exhibit morphological changes

Exposure to apatinib was responsible for inducing apoptosis and morphological changes of the HCC cells. The apoptotic cells had typical morphological changes, such as cytoplasmic shrinkage and membrane blebbing, when optically observed. Increased cytoplasmic vacuolation was also observed during the treatment. In the 6-month extensive establishment process, the HCC cells became resistant to apatinib and displayed a fibroblast-like spindle-shaped cell shape slowly. The resistant cell morphology became more drastic when the confluence of the cells increased and when the treatment continued after 48 hours post-subculture. Comparatively, the change in

the cell morphology is more obvious in PLC/PRF/5-AR cells than in MHCC-97L-AR cells. The difference may be explained by the observation that the MHCC-97L-AR cells gradually turned round-shaped with time when the apatinib dose increased during the treatment. Intriguingly, these cells were not apoptotic or dead as they did not exhibit the distinct apoptotic cell morphology or detach from the bottom of culture dish, as well as the cell confluence increased when treatment time progressed. The MHCC-97L-AR cells might be vulnerable, even the cells could survive and proliferate, when being maintained at 20μM of apatinib, as increasing number of the resistant cells resumed the 'resistant type' morphology when the apatinib dose was reduced to 10μM (data not shown). However, our resistant cell models were not maintained at 10μM as the final dose setting.

3.4.2. Apatinib-resistant HCC cells are more resistant to apatinib-induced apoptosis than the mock via expressing lower ratios of Bax/Bcl-2

Induction of apoptosis is one of the important approaches in cancer drug development. One of the cellular mechanisms that contribute to drug resistance is inhibition of apoptosis, which promotes cell survival during and after drug treatments. In the apoptosis assay using flow cytometry, it has been shown that compared with the mocks, the resistant HCC cells are more resistant to apatinib-induced apoptosis in response to the increased dose of the drug.

The intrinsic pathway of apoptosis is regulated by the B-cell lymphoma 2 (Bcl-2) protein family. The Bcl-2 family proteins control apoptosis at the mitochondria by regulating mitochondrial outer membrane permeabilization (MOMP) by multidomain pro-apoptotic proteins such as the Bcl-2-associated X protein (BAX) and the Bcl-2

homologous antagonist/killer (BAK). The MOMP results in the cytosolic release of pro-apoptotic mitochondrial intermembrane space proteins like cytochrome c, leading to caspase activation and apoptotic cell death. The Bcl-2 protein was highly upregulated in the apatinib-resistant human HCC cell lines established. The Bcl-2 is widely accepted as an anti-apoptotic gene. In cancer cells, overexpression of this cellular protein often blocks or delays onset of apoptosis, which reduces the cell sensitivity to apatinib and gives rise to the drug resistance.

The Bcl-2 protein has been shown to be associated with sorafenib resistance in HCC as significant increases in the Bcl-2 expression were observed in sorafenib-resistant human HCC cell lines (Tutusaus et al., 2018). In our apatinib-resistant human HCC cell models, we also observed a significant increase in the Bcl-2 expression. This observation agrees with the previous findings that the Bcl-2 expression is closely associated with drug resistance in the cancer cells. Although the Bax protein expression did not show a consistent trend in the resistant cell models, we could show that a low Bax/Bcl-2 ratio was a characteristic for apatinib-resistant cells and a high Bax/Bcl-2 ratio was characteristic for apatinib-sensitive cells. The ratio of Bax/Bcl-2 determines the susceptibility of HCC cells to apatinib-induced apoptosis and thus the apatinib resistance in HCC cells.

3.4.3. Apatinib resistance confers higher potentials for migration and invasion

The trans-well migration and invasion assays are used to analyze the ability of the apatinib-resistant HCC cells to directionally respond to chemo-attractants such as growth factors. In the assays, it has shown that the resistant cells have higher capabilities to migrate and invade. Migratory and invasive capabilities of cancer cells

are the key aspects of metastasis, which are mainly driven by cell motility (Le Devedec et al., 2010). The motility of cancer cells is dependent on the changes in cell morphology caused by dynamic modifications in the polymerization of Actin leading to rearrangements of the cytoskeleton (Vignjevic & Montagnac, 2008). The changes in cellular morphology and their impact on cell motility are associated with alternations among epithelial and mesenchymal phenotypes. The epithelial-to-mesenchymal transition (EMT) is often linked to morphological modifications, cytoskeleton remodeling, loss of tight junctions, and acquisition of migratory and invasive capacities (Valastyan & Weinberg, 2011). Recent evidence reveals that apatinib inhibits the invasion and metastasis of HCC cells thorough downregulation of calcium-dependent, zinc-containing matrix metalloproteinases (MMP)-related proteins, whose activities are post-translationally controlled by endogenous tissue inhibitors of metalloproteinases (TIMP), by regulating the nuclear factor kappa B (NF-κB) signaling pathway (He et al., 2020). The involvement of MMP has been frequently reported in activation of EMT leading to development of drug resistance in various cancer types. The question whether MMP would play a part in apatinib resistance in HCC should be addressed. Further investigation is needed to verify the relationship between apatinib resistance and EMT.

CHAPTER 4

IDENTIFICATION OF CYP1A1 AS A KEY PLAYER IN MEDIATING APATINIB RESISTANCE IN HCC

Section 4.1 Introduction

Acquired drug resistance is one of the major problems in chemotherapy and molecularly targeted therapy. The development of drug resistance in cancer cells during treatments often render the therapies ineffective as tumors being more drug-resistant relapse after the treatments and are associated with high morbidity and mortality. Understanding of how acquired drug resistance is mechanistically mediated in cancer cells is of utmost importance to develop novel medications and increase the applicability of drugs currently available in cancer treatments.

Although sorafenib has been approved by the United State Food and Drug Administration (USFDA) as the first standard drug for advanced HCC, the emergence of novel therapeutics has stagnated over a decade until the approval of lenvatinib in 2017. More importantly, recurrence is frequently observed in patients with advanced HCC after receiving sorafenib treatment. This indicates that HCC cells develop acquired resistance to sorafenib during or after repeated treatments. In recent years, orphan drugs are receiving much attention by drug scientists as an increasing body of evidence has proven them as potential promising medications against cancer. Apatinib is currently undergoing multiple clinical trials on HCC patients, but studies on the acquired resistance to apatinib in HCC are very insufficient. Current knowledge about the molecular mechanisms underlying apatinib resistance in cancer is little. Only a handful of studies have reported some key contributors mediating apatinib resistance in gastric cancer (Teng, Xu, Chen, et al., 2018; Teng, Xu, Lyu, et al., 2018). Unveiling the mechanisms mediating apatinib resistance can provide clearer knowledge about the drug interactions and more precise decision on prescribing apatinib to cancer patients.

The fact that acquired drug resistance is driven by single or multiple mechanisms in tumor cells critically limited the efficacy of cancer drugs. In order to create a global picture of cellular functions, we investigate the changes in the gene expression in the resistant cell lines by performing RNA sequencing. The use of RNA sequencing has the power to expedite the identification of drug resistance determinants and biomarkers in HCC cells. Based on the RNA sequencing data obtained, we performed the pathway analysis by using the Gene set enrichment analysis (GSEA) to study the pathways closely associated with the apatinib resistance acquired by the cells. Following identification and selection of the target gene candidates, the functional roles of the target gene in regulating apatinib resistance were characterized by gene knockdown approach and subsequent *in vitro* functional assays as well as by studying the upstream signaling components. These steps were essential for the selection of specific inhibitors and hence the development of drug combination regime with apatinib **(Figure 12)**.

Section 4.2 Experimental Scheme

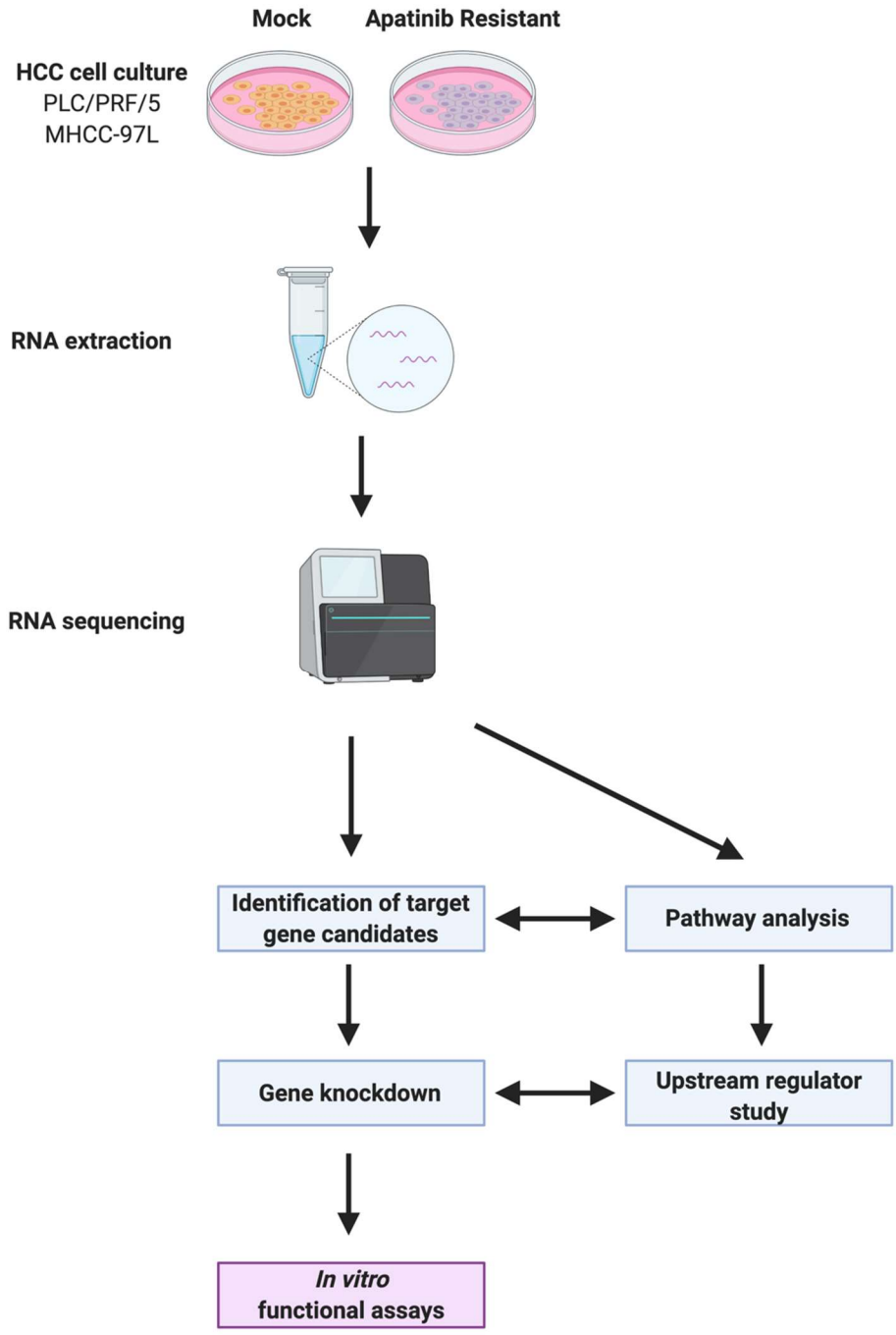

Figure 12. Illustrated experimental scheme for identifying the CYP1A1 as a key player in mediating apatinib resistance in HCC cells.

Section 4.3 Results

4.3.1. RNA sequencing revealed the CYP1A1 gene as the target candidate potentially having the closest association with apatinib-resistance in HCC cells

RNA sequencing was performed to profile the overall alternation of gene expression in PLC/PRF/5-AR and MHCC-97L-AR cells compared with their corresponding mock. The expression of approximately 5000-6000 genes were altered to different extent, with 4279 genes being commonly up-regulated (transcripts per million (TPM) fold change >1) and 2832 genes being commonly down-regulated (TPM fold change <1) in the two apatinib-resistant cell lines **(Figure 13A)**. A total of 23 genes were found to be ≥2-fold up-regulated (Log_2(TPM fold change) ≥1) commonly in both apatinib-resistant human HCC cell lines when compared with their corresponding DMSO-treated mock **(Table 2)**.

The cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) gene was target candidate that ranked the highest upregulated commonly in both established apatinib-resistant HCC cell lines. Following the CYP1A1, the second target candidate was the Chromosome 10 Open Reading Frame 90 (C10orf90) that ranked second in MHCC-97L (Log_2(TPM fold change) = 2.96) and fifth in PLC/PRF/5 (Log_2(TPM fold change) = 4.45) in response to the treatment **(Figure 13B)**. The C10orf90 gene currently is not well understood and whether this gene has association with drug resistance in HCC remains largely unknown. The C10orf90 encodes for the Fragile-site Associated Tumor Suppressor (FATS), an (E2-independent) E3 ubiquitin-conjugating enzyme, that is believed to function in centrosome formation.

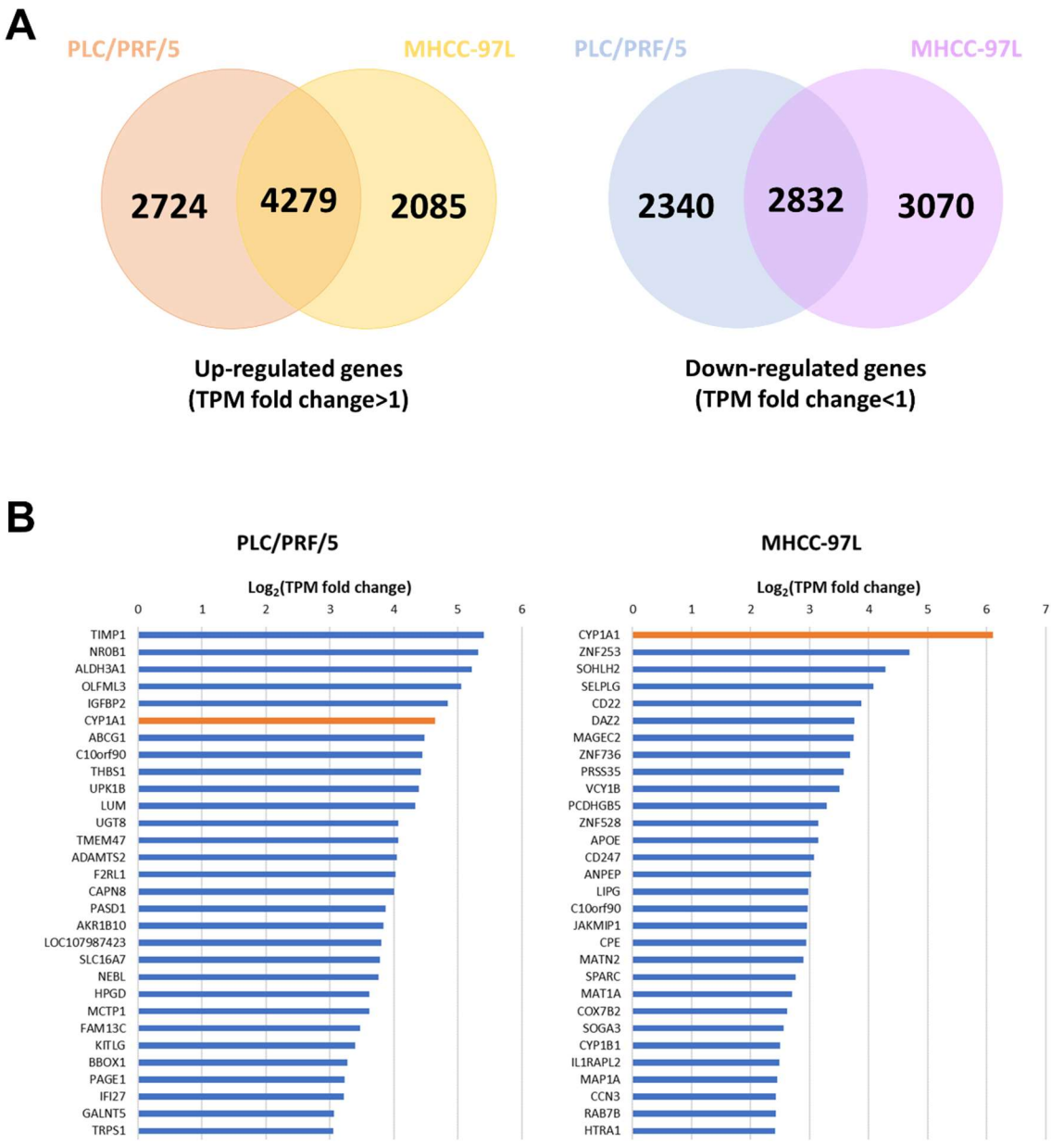

Figure 13. Metabolic functions and overall gene expression were altered in apatinib-resistant HCC cells. **(A)** Venn diagrams showing 4,279 genes being commonly up-regulated (TPM fold change >1) and 2,832 genes being down-regulated (TPM fold change <1) in PLC/PRF/5-AR and MHCC-97L-AR compared to their mock. **(B)** Top-ranked 30 genes up-regulated in apatinib-resistant PLC/PRF/5 and MHCC-97L cells compared with their corresponding mock cells. CYP1A1 ranked third in PLC/PRF/5 cells and first in MHCC-97L cells.

Table 2. A total of 23 genes found to be ≥2-fold up-regulated (Log_2(TPM fold change) ≥1) commonly in the two apatinib-resistant human HCC cell lines (PLC/PRF/5-AR and MHCC-97L-AR) when compared to their corresponding mock-treated (DMSO) cells by RNA sequencing. The function(s) of the gene or its association with disease(s) is tabulated accordingly.

	Genbank Accession Number	Gene Symbol	Gene name	Function(s)	Log_2(TPM fold change)	
					PLC/PRF/5	MHCC-97L
1	NM_000499.5	CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1	Xenobiotic metabolism, steroid hormone biosynthesis, tryptophan and retinol metabolism	4.64	6.10
2	NM_001004298.3	C10orf90	Chromosome 10 Open Reading Frame 90 / Fragile-site associated tumor suppressor	Centrosome formation	4.45	2.96
3	NM_001303040.2	ITGA10	Integrin Subunit Alpha 10	PI3K/Akt signaling, cell-extracellular matrix adhesion	2.64	2.40
4	NM_032461.4	SPANXB1	SPANX Family Member B1	Regulation of testis-specific gene transcription and translation	1.71	2.36
5	NM_004104.5	FASN	Fatty acid synthase	Fatty acid biosynthesis, AMPK and insulin signaling	1.06	2.22
6	NM_198461.3	LONRF2	LON Peptidase N-Terminal Domain And Ring Finger 2	Association with ATP-dependent peptidase	1.49	2.00
7	NM_015444.3	TMEM158	Transmembrane Protein 158	Association with ovarian carcinogenesis	1.40	1.95
8	NM_001286352.3	OLFML3	Olfactomedin Like 3	Pro-angiogenic factor	5.06	1.91
9	NM_030762.3	BHLHE41	Basic Helix-Loop-Helix Family Member E41	Circadian rhythm	1.40	1.86
10	NM_004915.3	ABCG1	ATP-binding cassette sub-family G member 1	ABC transporter	4.48	1.65
11	NM_003048.6	SLC9A2	Solute Carrier Family 9 Member A2	Na+/H+ exchanger	2.74	1.62
12	NM_001818.4	AKR1C4	Aldo-keto reductase family 1 member C4 / 3α-Hydroxysteroid dehydrogenase	Bile acid and steroid hormone biosynthesis	1.05	1.58
13	NM_001256566.2	F2RL2	Coagulation factor II receptor-like 2 / Protease activated receptor 3	Thrombin signaling	2.81	1.57
14	NM_001197181.2	TUBB3	Tubulin Beta 3 Class III	Microtubule formation	1.17	1.57
15	NM_001432.3	EREG	Epiregulin	MAPK, ErbB and PI3K/Akt signaling	1.70	1.49
16	NM_001199380.1	RNF145	Ring Finger Protein 145	Sterol-responsive ER-resident E3 ligase	1.10	1.40
17	NM_001277226.2	LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5	Wnt signaling	1.04	1.37
18	NM_002236.5	KCNF1	Potassium Voltage-Gated Channel Modifier Subfamily F Member 1	Ion channel	1.58	1.32
19	NM_001104554.1	PAQR5	Progestin And AdipoQ Receptor Family Member 5	Progesterone signaling	1.20	1.23

20	NM_001350001.2	JCAD	Junctional Protein Associated With Coronary Artery Disease	Association with CAD and myocardial infarction	1.42	1.18
21	NM_003311.4	PHLDA2	Pleckstrin Homology Like Domain Family A Member 2	Tumor suppressor	1.73	1.16
22	NM_000475.5	NR0B1	Nuclear Receptor Subfamily 0 Group B Member 1	Steroid hormone biosynthesis, SF1-mediated transcription	5.32	1.05
23	NM_001010860.2	SAMD15	Sterile Alpha Motif Domain Containing 15	Association with Visual Cortex Disease and Visual Pathway Disease	1.31	1.03

4.3.2. Up-regulation of CYP1A1 may be closely associated with cell cycle regulation in the apatinib-resistant HCC cells

Pathway analysis was conducted by using the RNA sequencing data and the GSEA software to investigate candidate pathways potentially associated with the apatinib resistance in PLC/PRF/5-AR and MHCC-97L-AR cells. The GSEA was computed by using the Molecular Signatures Database (MSigDB) C2 curated gene sets. Genes detected with TPM<1 were considered as too low to detect or undetectable and were excluded in the analysis. A total of top 15 gene sets were enriched in PLC/PRF/5-AR cells, whereas a total of top nine gene sets were enriched in MHCC-97L-AR cells **(Table 3)**. Four gene sets were commonly enriched in both cell lines PLC/PRF/5-AR and MHCC-97L-AR, including E2F targets (p<0.001), G2M checkpoints (p<0.001), as well as MYC targets V1 (p<0.001) and V2(p=0.002; p<0.001 respectively).

The E2F signaling was the top-ranked pathway commonly up-regulated in both apatinib-resistant HCC cell lines. The E2F family of transcription factors is most well-known for its ability to regulate the expression of genes required for DNA replication and cell cycle progression, as well as the transcription of upstream components of numerous signal transduction pathways including signaling associated with the Ras family, endothelial cell growth factor 1 (ECGF1), transforming growth factor α (TGFα), fibroblast growth factors, insulin-like growth factor 1 (IGF1), platelet-derived growth factors (PDGF), and VEGF (Androutsopoulos, Tsatsakis, & Spandidos, 2009). The expression of CYP1A1 is regulated by the Aryl Hydrocarbon Receptor (AhR) signaling pathway which is believed to be the most important regulatory mechanism of the CYP1A1. Previous studies have shown that the AhR signaling is able to induce other signaling pathway via protein interaction cross-links, such as the AhR-VEGF

pathway through the ATF4 (Terashima, Tachikawa, Kudo, Habano, & Ozawa, 2013). As the CYP1A1 is a downstream target of the AhR signaling, it may also have associations with these pathways via the components of AhR and E2F signaling.

The G2/M DNA damage checkpoint serves to prevent the cell from entering mitosis (M phase) with genomic DNA damage. It is believed that cancer cells are dependent on the G2/M checkpoints for repair of DNA damage, due to the presence of defective G1 checkpoint mechanisms. An early study (Santini, Myrand, Elferink, & Reiners, 2001) using murine hepatoma 1c1c7 cells revealed that the Cyp1a1 transcription was significantly suppressed in the cells arrested in G2/M phase, even pre-treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a strong AhR activator. Notably, the suppression reflected neither changes in the relative content of the proteins comprising the AhR complex nor a suppression of AhR activation and translocation to the nucleus, though the connections between the CYP1A1 and cell cycle regulation remain largely unknown.

The MYC family is a group of regulatory proteins, many of which is proven to be closely associated with carcinogenesis in the liver. A previous study (X. Yang et al., 2005) revealed that the AhR signaling constitutively repressed c-Myc transcription in human breast cancer Hs578T cells. Interestingly, the study documented that AhR from Hs578T cells did not bind to the endogenous CYP1A1 promoter region defined by the authors unless the cells were pre-treated with TCDD, suggesting a preferential gene tropism for constitutively active AhR.

Table 3. A list of the top 15 Molecular Signatures Database (MSigDB) hallmark gene sets (GS) enriched by the Gene Set Enrichment Analysis (GSEA) using the gene expression data of apatinib-resistant HCC cell lines from RNA sequencing. The GS commonly enriched in both cell lines are highlighted in red.

	PLC/PRF/5-AR		MHCC-97L-AR	
	MSigDB Hallmark GS	***p*-value**	**MSigDB Hallmark GS**	***p*-value**
1	E2F targets	<0.001	E2F targets	<0.001
2	G2M Checkpoint	<0.001	G2M Checkpoint	<0.001
3	MYC targets V2	0.002	MYC targets V2	<0.001
4	Epithelial mesenchymal transition	<0.001	MYC targets V1	<0.001
5	Angiogenesis	<0.001	Unfolded protein response	<0.001
6	Mitotic spindle	<0.001	MTORC1 signaling	<0.001
7	TNFA signaling via NFKB	0.01	Oxidative phosphorylation	<0.001
8	MYC targets V1	<0.001	DNA repair	0.044
9	Reactive oxygen species pathway	0.055		
10	Fatty acid metabolism	0.025		
11	TGF beta signaling	0.066		
12	Allograft rejection	0.071		
13	Coagulation	0.09		
14	Estrogen response early	0.063		
15	UV response UP	0.084		

4.3.3. CYP1A1 expression was elevated at both mRNA and protein levels in apatinib-resistant HCC cells

As CYP1A1 was found to be highly up-regulated in the apatinib-resistant HCC cells, it was selected as the study target for further investigation. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis demonstrated that the mRNA expression of the CYP1A1 was significantly up-regulated by 42-fold in MHCC-97L-AR cells ($p<0.01$) and by 21-fold in PLC/PRF/5-AR cells ($p<0.001$) when compared to the corresponding DMSO-treated mock counterparts **(Figure 14A)**. The extent of CYP1A1 upregulation seemed to be dependent on the cell line. In addition, elevated protein expression of the CYP1A1 was also verified by the Western blotting in both MHCC-97L-AR and PLC/PRF/5-AR cells with 2.8-fold and 1.5-fold increase respectively **(Figure 14B)**. Intriguingly, the fold increase in the endogenous CYP1A1 protein expression levels was much smaller than that in the mRNA expression levels. This deviation suggests that the turnover/degradation of CYP1A1 mRNA and protein and the miRNA control over the translation process could play a role in the complex milieu.

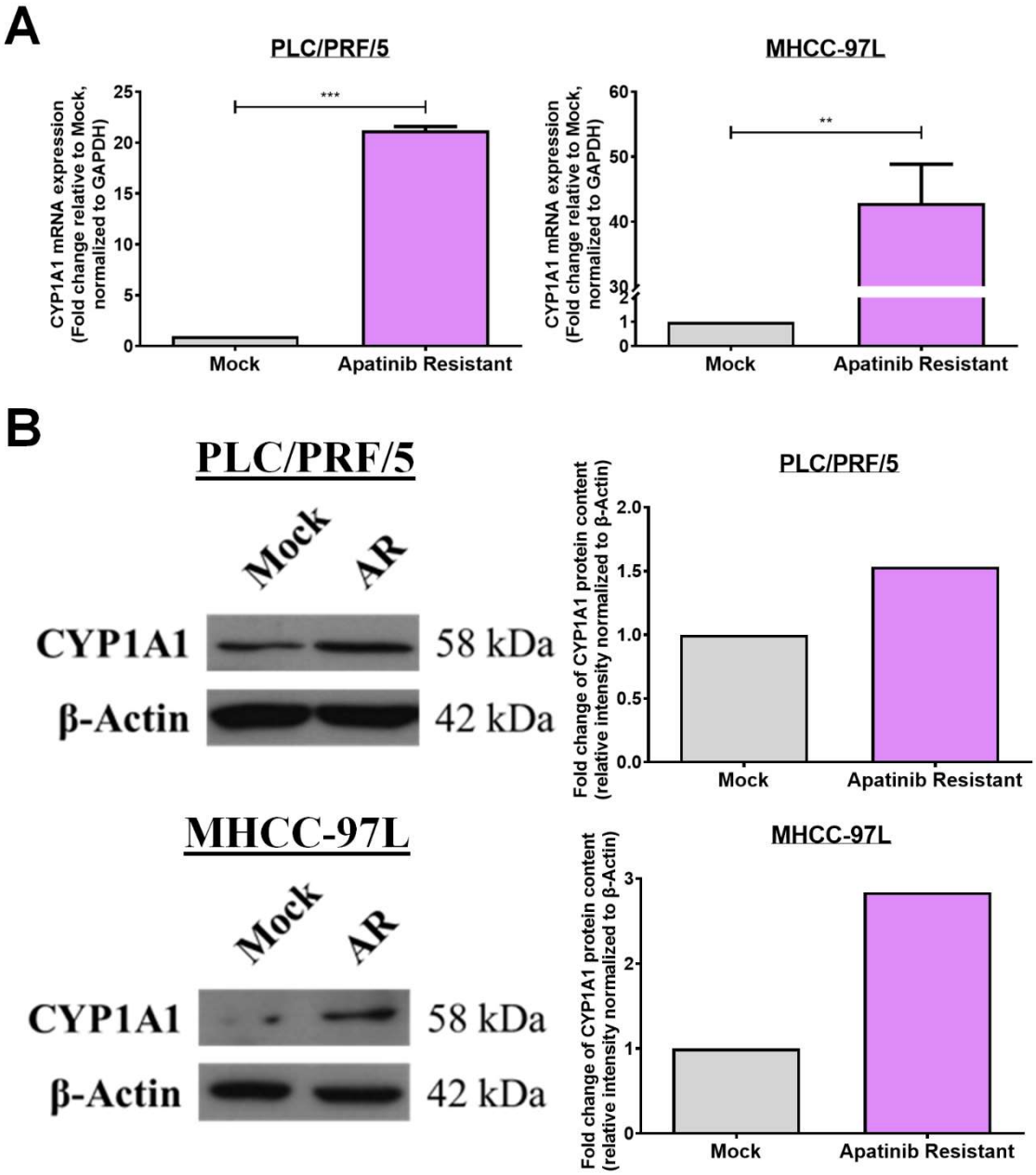

Figure 14. CYP1A1 was significantly up-regulated in apatinib-resistant HCC cells. **(A)** The mRNA expression was increased by 21-fold in PLC/PRF/5-AR cells (p<0.001) and by 42-fold in MHCC-97L-AR cells (p<0.01). Error bars represent the SD of three independent replicates. Student's t-test, **p<0.01; ***p<0.001. **(B)** The protein expression was increased by 1.5-fold in PLC/PRF/5-AR cells and 2.8-fold in MHCC-97L-AR cells.

4.3.4. Establishment of shCYP1A1 knockdown HCC cells

In order to examine the functional role of CYP1A1 in mediating apatinib resistance in HCC, CYP1A1-knockdown HCC cells were established by using lentivirus-based shRNA approach. Two knockdown clones, shCYP1A1#20 and shCYP1A1#21 were established for both PLC/PRF/5 and MHCC-97L cells. In these two cells, the two knockdown clones both displayed a significant reduction in the mRNA expression of the CYP1A1 gene. The knockdown efficiencies on the mRNA expression levels were approximately 50% relative to the non-target control (NTC) **(Figure 15A)**. The Western blot analysis showed that the knockdown efficiencies on the endogenous CYP1A1 protein expression in the two knockdown clones of PLC/PRF/5 were about 40% and 25% respectively, whereas the suppression effects in the two knockdown clones of MHCC-97L were approximately 90%. **(Figure 15B)**.

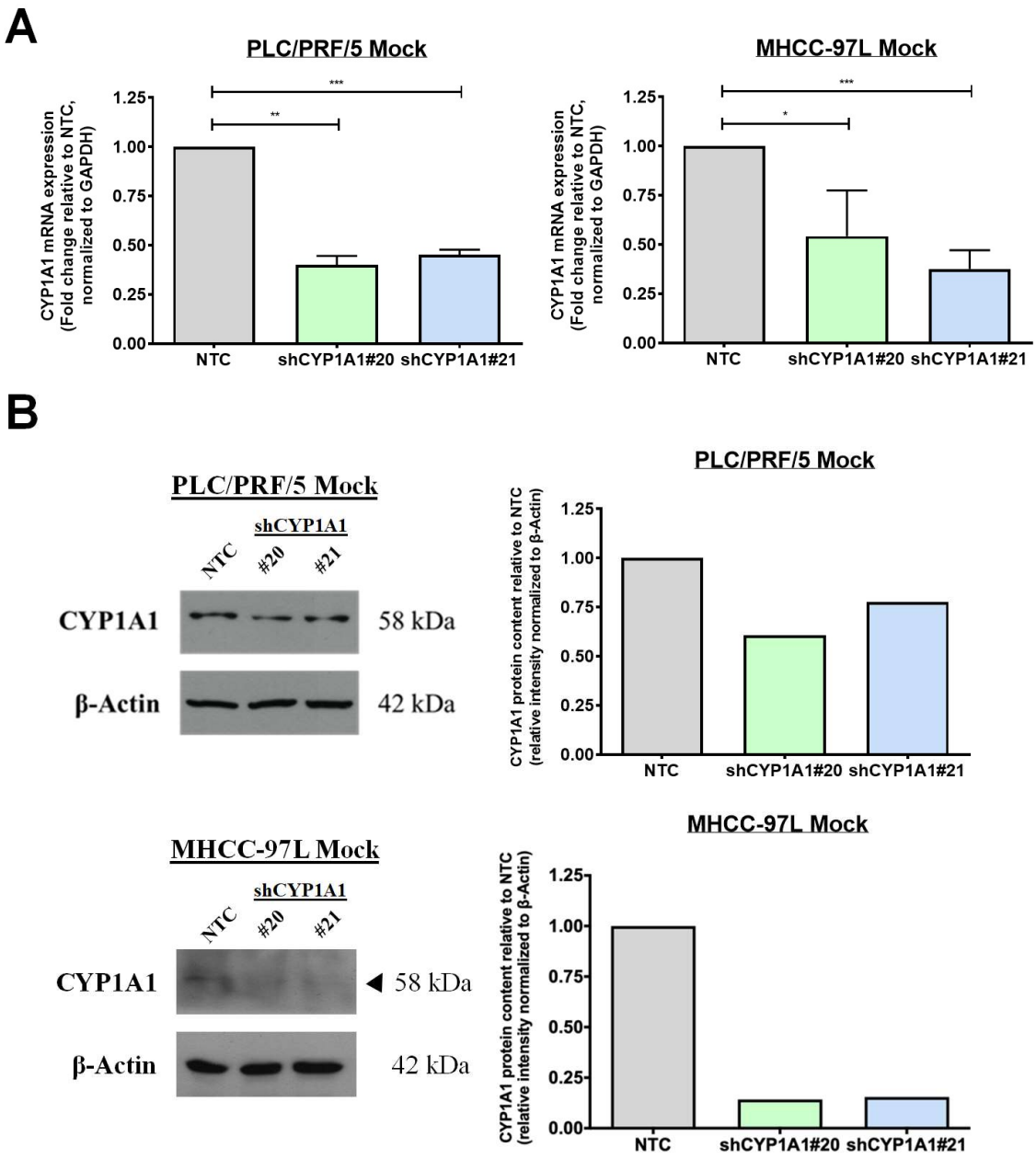

Figure 15. Endogenous CYP1A1 expression was reduced in the two shCYP1A1 knockdown clones. **(A)** The suppression effects on the mRNA level were approximately 50%. The experiments were repeated in triplicate for PLC/PRF/5 Mock and four times for MHCC-97L Mock. Data are shown as mean ± SD. Student's t-test, *p<0.05; **p<0.01; ***p<0.001. **(B)** The suppression effects on the protein levels were about 40% and 25% with respect to the shCYP1A1#20 and shCYP1A1#21 clones of PLC/PRF/5, whereas the protein levels were suppressed by approximately 90% in the two knockdown clones of MHCC-97L.

4.3.5. Knockdown of CYP1A1 sensitized HCC cells to apatinib treatment

It has been demonstrated in our previous data that CYP1A1 was significantly over-expressed in apatinib-resistant HCC cells. It is of interest to investigate whether suppression of endogenous CYP1A1 could increase the sensitivity of HCC cells to apatinib. The two shRNA-based knockdown clones, shCYP1A1#20 and shCYP1A1#21 were then employed to examine their sensitivity to apatinib by Annexin V/PI staining. The CYP1A1 knockdown enhanced the apoptosis of HCC cells upon apatinib treatment **(Figure 16)**. By comparison, the suppression in shCYP1A1#20 clone seemed to have a stronger sensitizing effect than that in shCYP1A1#21 clone in response to apatinib. The percentages of apoptotic cells were increased in shCYP1A1#20 clone by approximately 2-fold in PLC/PRF/5 Mock ($p<0.001$) and 1.25-fold in MHCC-97L Mock ($p<0.01$) compared to that in shCYP1A1#21 clone increased by approximately 1.4-fold ($p<0.05$) and 1.25-fold ($p<0.05$) respectively.

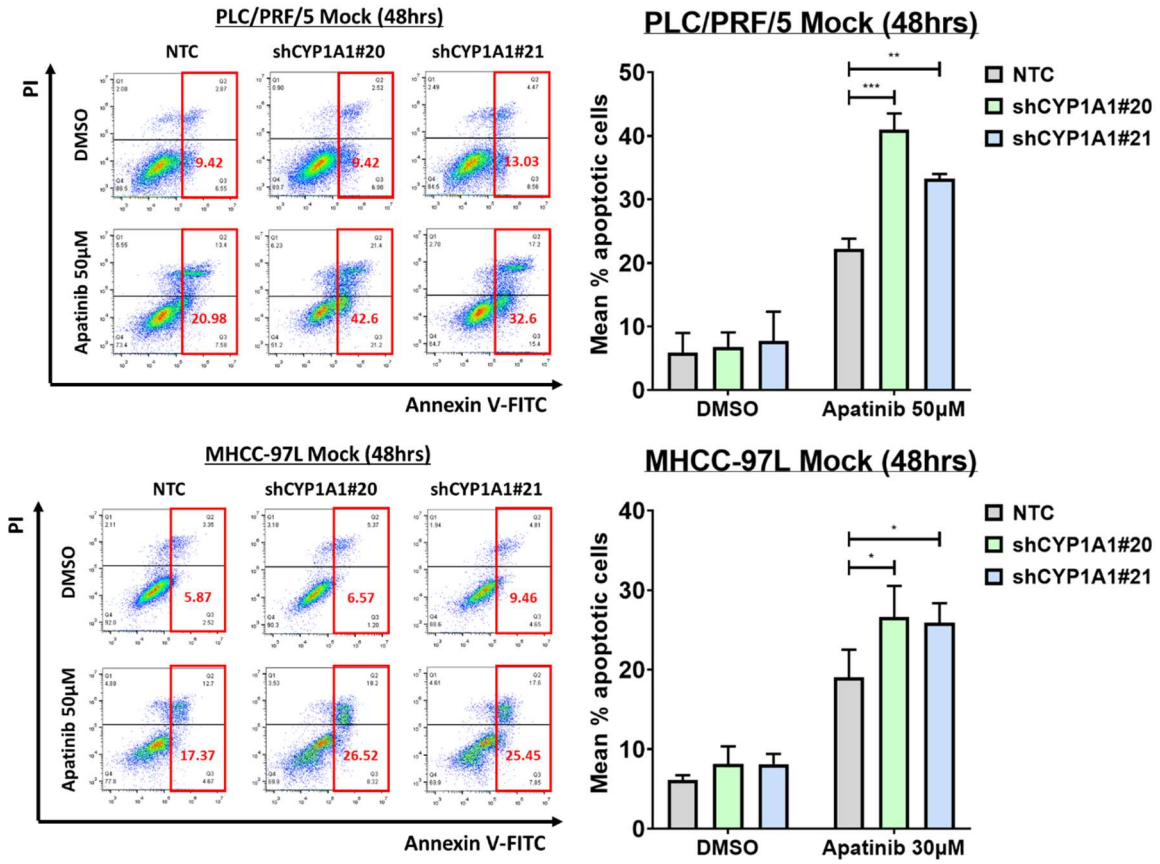

Figure 16. Knockdown of CYP1A1 increased the sensitivity of HCC cells to apatinib. For PLC/PRF/5 cells, the experiments were repeated in triplicate, whereas the experiments were repeated four times for MHCC-97L cells. Data in the bar charts are shown as mean ± SD. Student's t-test, *p<0.05; **p<0.01; ***p<0.001.

4.3.6. Knockdown of CYP1A1 reduced its enzymatic activity in HCC cells

Our data have previously showed that the expression levels of CYP1A1 were upregulated in the apatinib-resistant HCC cells. Apart from the expression, it is of interest to investigate whether the change in the expression would alter the activity of the enzyme. For this purpose, the enzymatic activity of the CYP1A1 in the apatinib-resistant cells was assessed **(Figure 17)**. In PLC/PRF/5-AR, the CYP1A1 activity was significantly increased by approximately 2.5-fold as compared with the Mock. In addition, knockdown of the CYP1A1 significantly reduced the enzymatic activity by about 60% as demonstrated in the two knockdown clones.

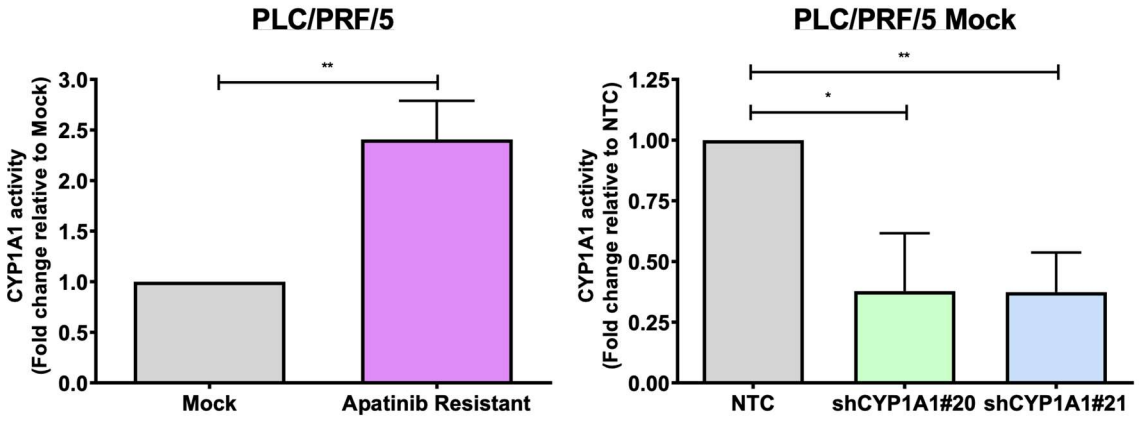

Figure 17. Enzymatic activity of CYP1A1 correlated with its expression levels in apatinib-resistant and CYP1A1-knockdown HCC cells. The experiments were repeated four times. Student's t-test, *$p<0.05$; **$p<0.01$.

Section 4.4 Discussion

In this chapter, the CYP1A1 gene was identified by RNA sequencing as the top-ranked gene in the apatinib-resistant human HCC cells. CYP1A1 has the highest fold change of up-regulation in the expression levels of transcripts as compared to other gene candidates on the sorted lists. The overexpression was verified by qPCR analysis and Western blotting. Therefore, we hypothesize that CYP1A1 may functionally mediate the apatinib resistance in HCC cells. To investigate the role of CYP1A1 in regulating apatinib resistance in the cells, lentiviral-based endogenous CYP1A1-knockdown HCC cell clones were first established from the DMSO-treated Mock HCC cells. The CYP1A1-knockdown clones were then treated with apatinib for 48 hours and significantly showed enhanced apoptosis rates compared with the NTC, providing pivotal evidence for CYP1A1 functioning in regulating apoptosis induced by apatinib. Subsequent enzymatic activity assay showed the increased CYP1A1 activity in apatinib-resistant HCC cells as well as the concurrent suppression of the basal activity in the CYP1A1-knockdown clones. In this discussion session, we comprehensively review the physiological functions of CYP1A1 and its pathological roles in cancer so as to shed light on its importance in the development of acquired resistance to apatinib in HCC.

4.4.1. Physiological roles and functions of CYP1A1

CYP enzymes are a superfamily of hemoproteins possessing monooxygenase activity. They are involved in metabolism of various endogenous compounds such as hormones, bile acids, and cholesterol, as well as xenobiotics including environmental pollutants and drugs. In human, CYP1A1 is an enzyme responsible for the biotransformation of polycyclic aryl hydrocarbons (PAH), aromatic amines, and polychlorinated biphenyls into polar compounds which thereby can be conjugated to soluble compounds being excreted in urine or bile. Nevertheless, under certain circumstances, this enzyme catalyzes the bioactivation of reactive species capable of DNA adduct formation leading to mutagenesis. The human CYP1A1 gene, being 6,069 bp in length, is located at the CYP1A1_CYP1A2 locus on the chromosome 15q24.1, which shares a regulatory region of 23,306 bp with the CYP1A2 gene that is oriented in opposite direction (Z. Jiang et al., 2005). The 5′ flanking region is shared by both genes and is comprised of a bidirectional promoter and multiple xenobiotic responsive elements (XRE), that activate and regulate the gene expression of CYP1A1 and CYP1A2 (Jorge-Nebert et al., 2010).

The human CYP1A1 enzyme has a molecular weight of 58.16 kDa and consists of 512 amino acids. The first 30 amino acid residues of its N-terminal region allow the enzyme to associate with the mitochondrial membrane and the disordered region of the smooth endoplasmic reticulum rich in unsaturated fatty acids for anchorage, unlike the human CYP1A2 which is mainly positioned in the ordered regions rich in cholesterol, sphingomyelin and saturated fatty acids (Park, Reed, Brignac-Huber, & Backes, 2014). This specific membrane localization is governed by the sequence motifs located at the N-terminal region (Park, Reed, & Backes, 2015). As the interactions among CYP1A1

and CYP1A2 occur in a membrane environment, the lipid membrane serves as an integral component mediating the functioning of P450 system. The proximal side of the P450 enzymes faced the aqueous environment, whereas the distal side was associated with the membrane-water interface. Substrate was modeled to approach the active site from the membrane through channels (Scott et al., 2016). Moreover, these N-terminal residues also mediate the interaction with the NADPH-CYP reductase which is a di-flavoprotein responsible for the electron transfer to naturally occurring electron acceptors such as the CYP enzymes and cytochrome-b_5.

In addition to its importance in xenobiotic metabolism, CYP1A1 is also involved in the metabolism of various endogenous molecules including arachidonic acid, eicosapentaenoic acid, and hormones like 17β-estradiol and melatonin. The endogenous and exogenous substrates of CYP1A1 is listed with corresponding enzymatic reaction in **Table 4**. Arachidonic acid and eicosapentaenoic acid are transformed by CYP1A1 to 14,15-epoxyeicosatrienoic acid and 17, 18-epoxyeicosatetraenoic acid, two signaling intermediates influencing cardiovascular pressure, respectively.

Table 4. CYP1A1 interacts with a vast number of endogenous and exogenous compounds.

Origin	Category compound	Type of reaction	References
Synthetic compounds	PAH	Oxidation epoxidation	(Shimada, Oda, Gillam, Guengerich, & Inoue, 2001)
	Nitrosamides	Nitroreduction	(Miyazaki, Sugawara, Yoshimura, Yamazaki, & Kamataki, 2005)
	Arylamines	*N*-hydroxylation oxidation	(Shimada et al., 2001)
	Benzotriazole	Oxidation	(Seaton, Schlosser, Bond, & Medinsky, 1994)
	Heterocyclic amines	*N*-hydroxylation oxidation	(Shimada et al., 2001)
	Nitroarenes	Nitroreduction	(Marumoto, Oda, & Miyazawa, 2011)
	Azoaromatic amines	Oxidation	(Shimada et al., 2001)
Natural compounds	Difuranocumarin	Epoxidation oxidation	(Shimada, Martin, Pruess-Schwartz, Marnett, & Guengerich, 1989)
	Nefrotoxin	Hydroxylation	(Stiborova, Hajek, Frei, & Schmeiser, 2001)
	Flavonoid	Hydroxylation *O*-demethylation	(Silva et al., 1997)
Drugs	Ellipticin	Oxidation	(Aimova et al., 2007)
	Omeprazol	Not determined	(Backlund, Johansson, Mkrtchian, & Ingelman-Sundberg, 1997)
	Oltipraz	Not determined	(I. J. Cho & Kim, 2003)
	Etoposide	Not determined	(Willis et al., 2018)
	Ellipticine	Not determined	(Willis et al., 2018)
	Bortezomib	Not determined	(Xu et al., 2012)
	Imatinib	Not determined	(Rochat et al., 2008)
	Osimertinib	Hydroxylation, demethylation	(MacLeod et al., 2018)
	Ponatinib	Hydroxylation	(Lin, Kostov, Huang, Henderson, & Wolf, 2017)
	Granisetron	Hydroxylation	(Lang, Radtke, & Bairlein, 2019)
	Riociguat	Hydroxylation	(Lang et al., 2019)
Endogenous substrates	Arachidonic acid	Hydroxylation	(Schwarz et al., 2004)
	Melatonin	Hydroxylation	(Ma, Idle, Krausz, & Gonzalez, 2005)
	Eicosapentaenoic acid	Epoxidation	(Schwarz et al., 2004)
	Stradiol	Hydroxylation	(Lee, Cai, Thomas, Conney, & Zhu, 2003)

4.4.2. Pathological Roles of CYP1A1 in Cancer

Numerous studies have evaluated the association between CYP1A1 polymorphism and cancer risk, since CYP1A1 is involved in the biotransformation of tobacco-derived PAH into carcinogenic metabolites, acting as an important modulator of the carcinogenic effect of PAH. For its frequent link with smoking-induced cancers, CYP1A1 polymorphism was first and extensively studied in lung cancer. Subsequent research also identified several types of CYP1A1 polymorphism in other cancer types such as breast, cervical, prostate, oral, as well as head and neck cancers. CYP1A1 is a polymorphic gene located at the chromosome 15q24.1 with 7 exons and 6 introns, of which 10 variant alleles in addition to the wild-type allele have been identified in humans. Among these genotypes, two single nucleotide polymorphism (SNP) variant alleles are frequently associated with the cancer risks, including the *Msp*I variant genotype, which has a *Msp*I restriction recognition site (5'-CCGG-3') in the intron 6, and the $Ile^{462}Val$ variant genotype, which has an isoleucine to valine change in the heme-binding region of the enzyme (Hirvonen, Husgafvel-Pursiainen, Karjalainen, Anttila, & Vainio, 1992). The polymorphic distributions seem to vary between smokers and non-smokers (Ezzeldin et al., 2017), and among ethnic groups (Inoue, Asao, & Shimada, 2000) in addition to cancer types.

Tobacco smoking is one of the risk factors for HCC. It was reported that smoking more than 25 cigarettes per day increased the risk of HCC by 55% (Petrick et al., 2018). The epidemiological relationship may help us to recall the association between CYP1A1 polymorphism and the risk of HCC. An early study has demonstrated the correlation between CYP1A1 3801 T>C gene polymorphism and an increased risk of HCC among Asian smokers (M. W. Yu et al., 1999). Although the specific

association is still inconclusive, a growing body of evidence recently suggests that the common SNP variants of CYP1A1 gene, involving the *Msp*I and Ile^{462}Val genotypes, may increase the susceptibility to HCC in the Han Chinese population (R. Li et al., 2009; B. W. Yu et al., 2015; L. Yu et al., 2012). While it is unclear how these gene mutations could affect the transcription and translation, it is hypothesized that the polymorphism would lead to changes in the expression and activity levels of CYP1A1, which alters metabolism and clearance of carcinogens and in turn causes variation in the cancer risk.

Despite its genetic contributions to cancer risk being controversial, the pathological role of CYP1A1 in cancer development and progression remains largely unknown. It was first reported that CYP1A1 regulated the progression of breast cancer *in vitro*, with evidence revealing a possible role of CYP1A1 in promoting the proliferation and survival of breast cancer cells through suppression of the AMP-activated protein kinase (AMPK) signaling (Rodriguez & Potter, 2013). The authors wrote that CYP1A1 silencing activated the AMPK phosphorylation and concomitantly down-regulated the phosphorylation of ERK1/2 and AKT, hypothesizing that CYP1A1 could be a possible upstream regulator of these kinases. However, no subsequent studies were ever conducted to investigate the oncogenic role of CYP1A1 in the development of breast and other cancers, and the association between CYP1A1 and cancer-related genes and pathways remains unclear. More comprehensive studies are required to clarify the pathological contributions of CYP1A1 to cancer development.

Uncontrolled cell growth and proliferation is a hallmark of cancer and is often attributed to dysregulated control of the cell cycle. Not only participating in

tumorigenesis, the cell cycle also plays a part in mediating drug resistance in cancer cells (Otto & Sicinski, 2017; Shah & Schwartz, 2001). Early studies have documented a mysterious connection between CYP1A1 and the cell cycle as variation of the CYP1A1 expression level in different phases of the cell cycle was repeatedly observed *in vitro* (Hamouchene, Arlt, Giddings, & Phillips, 2011; Santini et al., 2001). In addition, it was found that colchicine and nocodazole, two well-known microtubule interfering agents inducing G2/M cell cycle arrest, reduced the expression of CYP1A1 at both mRNA and protein levels while disrupting cytoskeleton integrity in murine hepatoma 1c1c7 cells and human HCC HepG2 cells (Dvorak et al., 2006; Scholler, Hong, Bischer, & Reiners, 1994). Microtubule dynamics change dramatically during the cell cycle, as reorganization of cytoskeleton composed of microtubules is crucial to cell division, which is responsible for formation of mitotic spindle and segregation of duplicated chromosomes into two daughter cells (Heng & Koh, 2010; Nakaseko & Yanagida, 2001). Alternation in CYP1A1 expression concurrent with the dynamics may indicate a possible mechanism contributing to drug resistance in cancer cells. Moreover, the G2/M phase may have influence on the transcriptional activity of AhR, as microtubule disarray suppresses the AhR transcriptional activity and thus the expression of the downstream target CYP1A1 (Dvorak, Ulrichova, & Modriansky, 2005). In this study, our pathway analysis using the GSEA program presents that cell cycle regulation including the G2/M checkpoints is a significant signaling pathway mediating apatinib resistance in HCC cells ($p<0.001$). Further, the E2F signaling is also identified to be significantly related to the resistance ($p<0.001$). It is well-known that the E2F family of transcription factors cooperates with regulation of cell cycle progression involving the G2/M checkpoints (Cuitino et al., 2019; Giangrande et al., 2004; Ren et al., 2002). Our finding is in harmony with the hypothesis that the underlying mechanism of

apatinib resistance in HCC cells is associated with the cell cycle regulation, in particular the G2/M checkpoint machinery.

4.4.3. Roles of CYP1A1 in drug resistance in cancer

One of the challenges in cancer treatments is to overcome drug resistance. Researchers have undertaken extensive efforts on developing strategies to combat drug resistance over the past decades. It is known that CYP1A1 is detected mainly in the extrahepatic tissues in small amounts under physiological conditions. A few studies report the important roles of CYP1A1 in metabolism of cancer drugs in conjunction with development of acquired resistance to the drugs in some cancer types **(Table 5)**. CYP1A1 not only is directly involved in metabolism of the drugs, but also induces other signaling pathways to support the resistance, while its direct and indirect effects on acquired drug resistance seem to be dependent on the type of cancer and the nature of drug. Until recently, CYP1A1 has not been considered to play a significant role in metabolic clearance of drugs in the liver because of its comparatively low intrahepatic expression with regard to its extrahepatic expression and compared to other CYP enzymes in intrahepatic tissues. A recent publication using mass spectrometry first demonstrates that intrahepatic CYP1A1 is the key CYP enzyme metabolizing granisetron, a serotonin 5-HT3 receptor antagonist, and riociguat, a stimulator of soluble guanylate cyclase approved for hypertension, with outstanding selectivity and efficiency, providing evidence for the clinically observed interindividual variability in their pharmacokinetics (Lang et al., 2019). Our data present a significant CYP1A1 up-regulation in HCC cells when being resistant to apatinib. Therefore, the importance of CYP1A1 in cancer drug discovery and development needs to be reconsidered.

Table 5. Studies on the association between the CYP1A1 up-regulation and drug resistance in some types of cancer.

Type of cancer	Drug resistance	Associated mechanism(s)	Reference
NSCLC	Gefitinib	Drug metabolism	(Alfieri et al., 2011)
NSCLC	Osimertinib	Drug metabolism	(MacLeod et al., 2018)
Breast	Doxorubicin	AhR/CYP1A1 pathway-mediated inhibition of PTEN and activation of β-catenin and Akt in CSCs	(Al-Dhfyan, Alhoshani, & Korashy, 2017)
Multiple myeloma	Bortezomib	Dll1/Notch activation	(Xu et al., 2012)
Chronic myeloid leukemia	Ponatinib	Drug metabolism and glutathione conjugation	(Lin et al., 2017)

CHAPTER 5

ELUDICATION OF MOLECULAR PATHWAYs BY WHICH CYP1A1 MEDIATES APATINIB RESISTANCE IN HCC

Section 5.1 Introduction

In previous chapters, we have explored that CYP1A1 is significantly overexpressed in apatinib-resistant HCC cells, which may underline the development of acquired resistance to apatinib in HCC that affects the treatment efficacy and the prognosis of patients. Through the establishment of CYP1A1 knockdown clones, CYP1A1 was shown to be significantly associated with apatinib resistance by controlling apoptosis upon treatment with the drug. In addition, we also presented that the overexpression of CYP1A1 could have a close relationship with cell cycle regulation including G2/M checkpoints, E2F and MYC signaling pathways, though we had not illuminated the detailed molecular mechanism coupling with the functional relationship between CYP1A1 and the cell cycle. In order to elucidate the mechanism mediating apatinib resistance in HCC, we hope to explore possible pathways associated with CYP1A1, cell cycle regulation and/or other signaling pathways that may contribute to the drug resistance.

In humans, CYP1A1 is expressed to a lesser extent under physiological conditions but is highly inducible upon stimulation via multiple intracellular signaling pathways. The regulatory signaling extensively studied with CYP1A1 expression and functions is the ligand-dependent aryl hydrocarbon receptor (AhR) pathway, whose activation can be induced by a vast number of environmental pollutants like dioxins, in particular 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and PAH, regulating a number of downstream enzymes and transporters, such as the CYP family members and the ABC transporters, that facilitate the biotransformation and elimination of these chemicals. For this reason, the AhR pathway is best characterized for its role in xenobiotic metabolism since its discovery.

Upon ligand binding, AhR dissociates from its regulatory proteins involving HBV X-associated protein 2 (XAP2), p23, and heat shock protein 90 (HSP90), and translocates to the nucleus. AhR contains a nuclear localization signal located in the N-terminal whose phosphorylation at either Ser-12 or Ser-36 residue inhibits its nuclear import, while phosphorylation of tyrosine residues in the C-terminal is required for the formation of a functional complex with aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR/ARNT heterodimer subsequently binds to the XRE positioned in the promoter region of AhR downstream targets like CYP1A1, and the interaction is inhibited by the heterodimer of ARNT with aryl hydrocarbon receptor repressor (AhRR). Transcription of the CYP enzymes is initiated concomitantly via interaction of several transcription factors such as Sp1 and co-activators like p300 and p/CIP, which binds with TATA binding protein (TBP) and the AhR complex, eventually recruiting RNA polymerase II for transcription. The AhR signaling is strictly controlled as prolonged activation has deleterious effects such as tumor induction and impacts on immune system (Andersson et al., 2002; Veldhoen et al., 2008). Intriguingly, since AhR recognizes xenobiotics and many natural compounds such as tryptophan metabolites, dietary components and microbiota-derived factors, the downstream enzyme CYP1A1 seems to have feedback regulation on the AhR signaling, which is important to curtail the duration of the signaling, by controlling ligand availability through oxygenation, leading to their metabolic clearance and detoxification, and thereby activation of the pathway (Schiering et al., 2017).

A causal link between overexpression of AhR/CYP1A1 and promotion of various cancer types has been established. Although the detailed mechanisms are still little understood, recent evidence more clearly reveals that the AhR/CYP1A1 pathway

may have a role in promoting doxorubicin resistance through inhibition of PTEN and activation of β-catenin and AKT in breast cancer stem cells (CSC) (Al-Dhfyan et al., 2017). The AhR/CYP1A1 pathway also facilitates metastasis by inducing the biosynthesis of 12(S)-hydroxyeicosatetraenoic acid (HETE) using a co-culture model of breast cancer MDA-MB231 cell spheroid with lymph endothelial cell (LEC) monolayer (Nguyen et al., 2016). Our study has identified CYP1A1 as a potential key player controlling HCC cell susceptibility to apatinib, but little is known about the molecular mechanisms of CYP1A1 contributing to the drug resistance. In this chapter, the potential mechanisms of how CYP1A1 may promote apatinib resistance in HCC were explored by RNA sequencing coupled with pathway analysis.

Section 5.2 Experimental Scheme

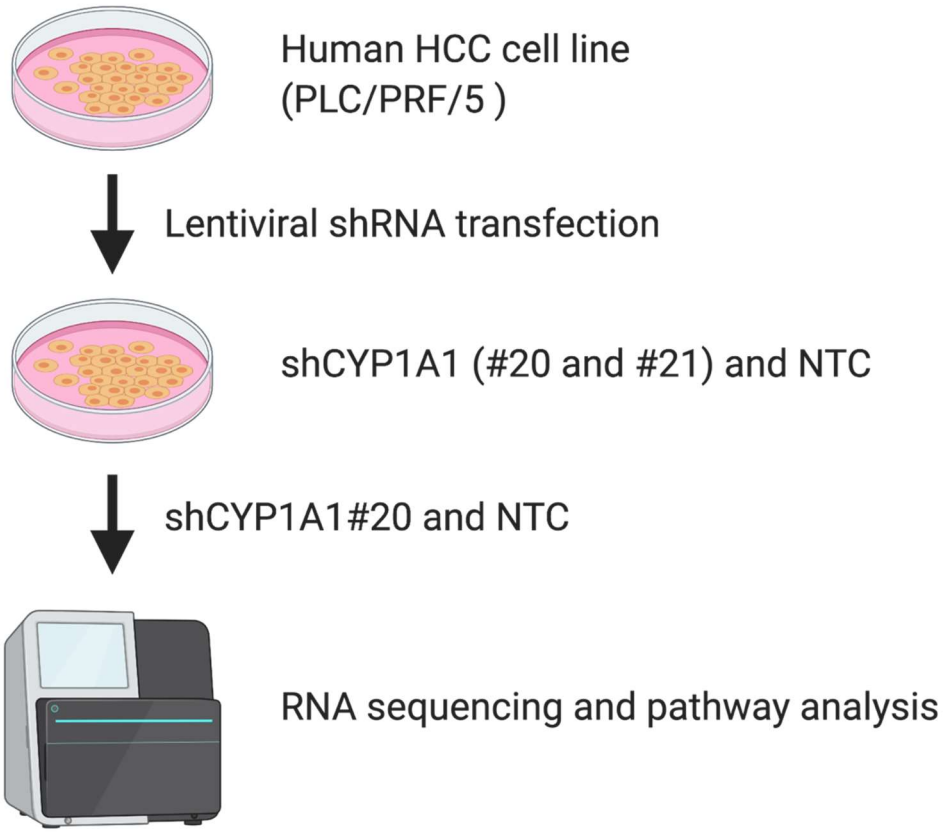

Figure 18. Illustrated experimental scheme for elucidating the molecular pathways of CYP1A1 mediating apatinib resistance in HCC cells using siRNA-based gene knockdown approach.

Section 5.3 Results

5.3.1. Knockdown of CYP1A1 down-regulated xenobiotic and bile acid metabolism in PLC/PRF/5 cells

PLC/PRF/5 shCYP1A1#20 cells were employed to perform RNA sequencing and subsequent GSEA for uncovering the regulatory mechanism of apatinib resistance contributed by CYP1A1. A total of 5,743 genes showed down-regulation, while 5,909 genes showed up-regulation in the CYP1A1-knockdown cells. To investigate the role of CYP1A1 in apatinib resistance in HCC cells for combating the resistance, possible pathways showing suppression in response to the gene knockdown were focused. Two gene sets from the MSigDB Hallmark database were significantly enriched in the analysis, namely xenobiotic metabolism (p<0.01; false discovery rate (FDR) q-value=0.114) and bile acid metabolism (p<0.05; FDR q-value=0.282) as shown in the enrichment plots **(Figure 19)**. Notably, some down-regulated gene set members of xenobiotic metabolism involved in the core enrichment included epoxide hydrolase 1 (EPHX1), UDP-glucose 6-dehydrogenase (UGDH), and 6-phosphogluconate dehydrogenase (6PGD), whereas those down-regulated GS members of bile acid metabolism involved neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4), farnesoid X receptor (NR1H4/ FXR), and ATP-binding cassette (ABC) transporter family members like ABCA3 and ABCD1.

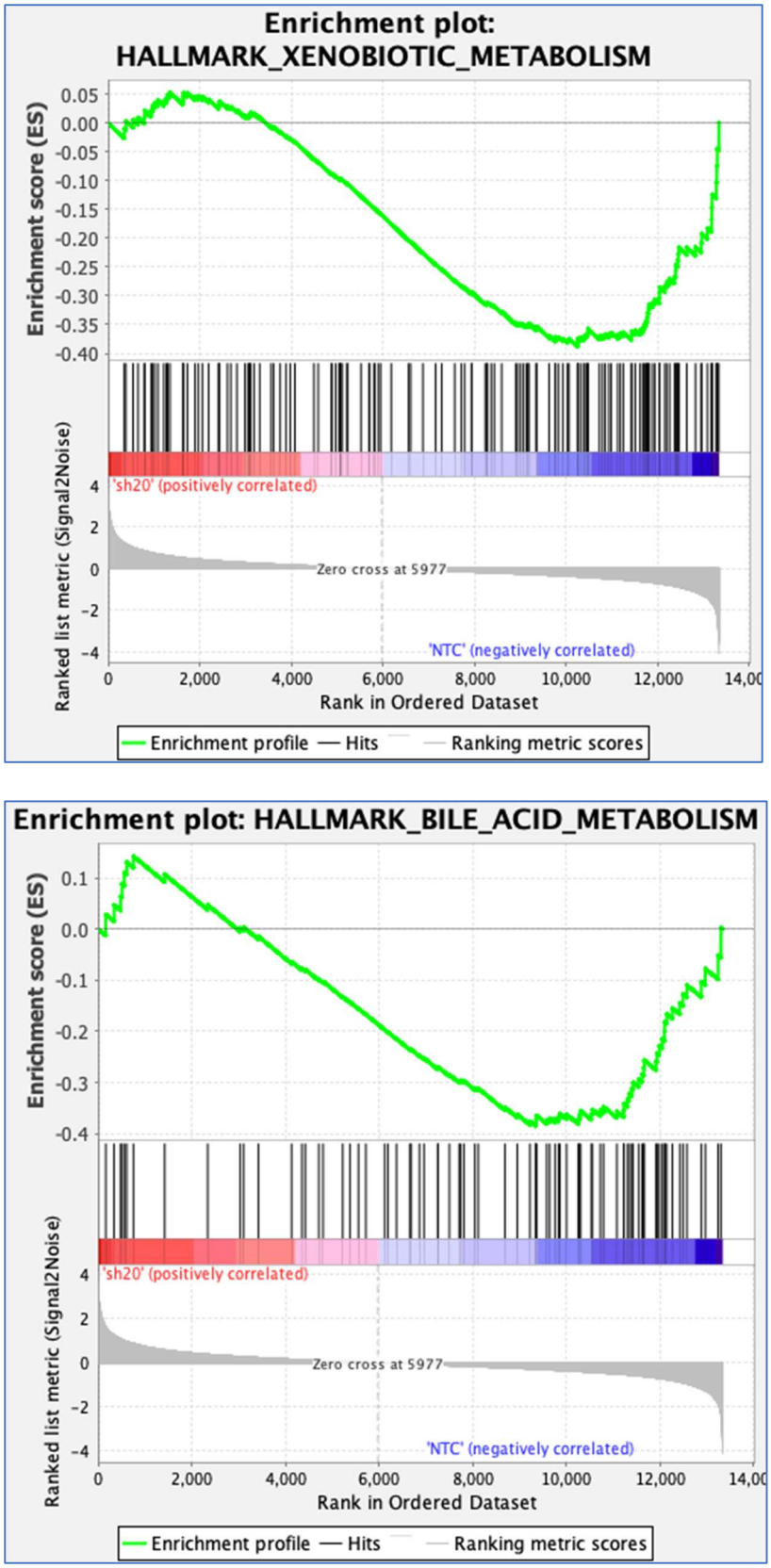

Figure 19. GSEA Enrichment plots of xenobiotic and bile acid metabolism in CYP1A1-knockdown PLC/PRF/5 cells.

5.3.2. Suppression of CYP1A1 expression correlated with down-regulation of RhoGDI signaling, SPINK1 pancreatic cancer pathway, and calcium signaling

Correlation with possible signaling pathways was further explored by employing the sequencing results of PLC/PRF/5 shCYP1A1#20 cells in the Ingenuity Pathway Analysis (IPA) software **(Figure 20)**. The negative and positive activation Z-scores represent suppression and activation of the particular signaling pathway respectively. It was found that knockdown of CYP1A1 expression had significant correlation (p<0.05) with the RhoGDI signaling, SPINK1 pancreatic cancer pathway, calcium signaling, and osteoarthritis pathway. Among the down-regulated pathways, RhoGDI signaling showed the most suppression with the lowest activation Z-score (Z=-1.604), followed by SPINK1 pancreatic cancer pathway (Z=-1) and calcium signaling (Z=-0.775) in the knockdown cells.

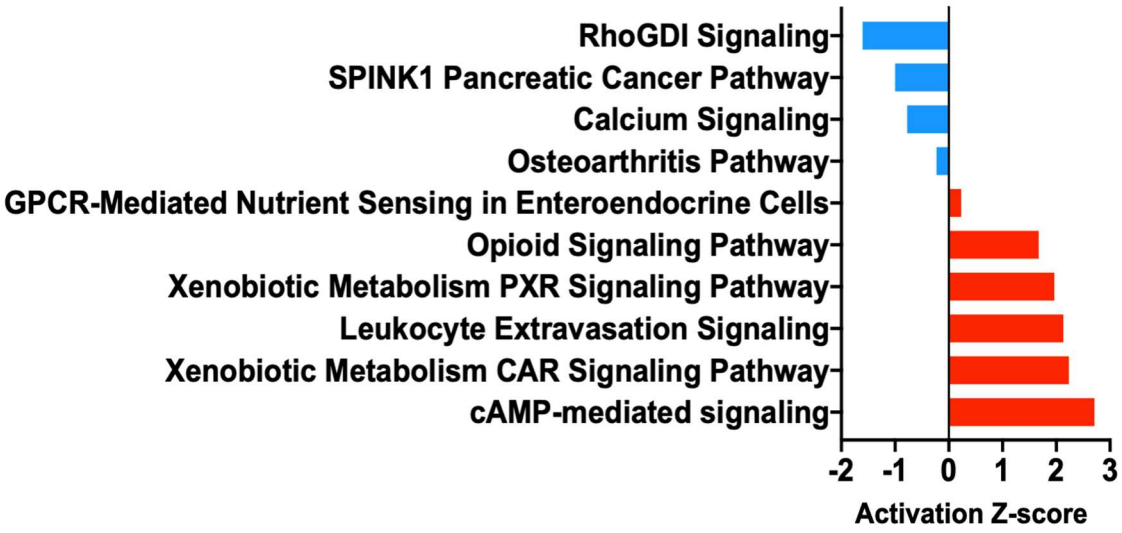

Figure 20. Alternation in activity of signaling pathways most down-regulated and most up-regulated with CYP1A1 knockdown in PLC/PRF/5 cells was shown. Down-regulation is regarded as activation Z-score<0 and indicated by a blue bar, whereas up-regulation is regarded as activation Z-score>0 and indicated by a red bar.

Section 5.4 Discussion

In the previous chapter, we have performed transient knockdown of CYP1A1 and demonstrated that suppression of CYP1A1 expression and activity increased the sensitivity to apatinib in HCC cells. We have also presented a comprehensive review on the functional and pathological roles of CYP1A1 in cancer. The association between CYP1A1 and apatinib resistance in HCC is being unraveled, though it is not understood how CYP1A1 is molecularly dysregulated in the apatinib-resistant HCC cells. In this chapter, to investigate the detailed mechanisms that are possibly involved in the regulation of CYP1A1, we employed the CYP1A1-knockdown clone shCYP1A1#20, whose suppression displayed stronger sensitizing effects to apatinib, to undergo RNA sequencing and pathway analysis.

It is well known that CYP1A1 is regulated by the AhR signaling pathway. Despite the induction by toxins and xenobiotics, CYP1A1 can also be regulated by tryptophan metabolites (Y. Cheng et al., 2015; Wei, Bergander, Rannug, & Rannug, 2000) and serotonin (Manzella et al., 2018) via the tightly controlled AhR signaling, as well as the Wnt/β-catenin signaling independent of the AhR control. Interestingly, cells expressing lower β-catenin activity (28%) requires higher doses of TCDD (10-50 nM) for induction of CYP1A1 expression, while those expressing 100% of β-catenin activity can display strong and saturating expression of CYP1A1 already at 5 nM TCDD (Schulthess et al., 2015), and it seems that there is an interplay between the AhR-mediated CYP1A1 induction and the Wnt/β-catenin signaling. Besides, some studies have proposed the implication of Src tyrosine kinase in the regulation of CYP1A1, though the precise mechanism needs to be elucidated. Moreover, nuclear receptors are also involved in the regulation of CYP1A1. Several studies have documented the

regulation of CYP1A1 expression by retinoic acid receptors/retinoid X receptors (RAR/RXR) since a retinoic acid responsive element (RARE) sequence is located within the CYP1A1 promoter (Fallone et al., 2004). It has been shown that low doses of RA can slightly induce cutaneous expression of CYP1A1 (Vecchini et al., 1994) , but little is known about the details of this pathway in regulating CYP1A1 expression in cancer including HCC. Nonetheless, CYP1A1 is negatively regulated by miR-892a-mediated post-transcriptional modification through the binding of miR-892a at a specific sequence located in the 3'-untranslated region of CYP1A1(Choi et al., 2012) . Collectively, these findings depict the CYP1A1 regulatory network.

5.4.1. Knockdown of CYP1A1 down-regulated xenobiotic and bile acid metabolism in HCC cells

We have employed PLC/PRF/5 shCYP1A1#20 and its mock counterpart cells in RNA sequencing and the GSEA and have explored that two gene sets, xenobiotic and bile acid metabolism, from the MSigDB Hallmark database could show significant down-regulation in the resistant phenotype compared to the parental phenotype. We reveal some notably down-regulated genes including EPHX1, UGDH, and 6PGD involved in the xenobiotic metabolism gene set, and NEDD4, FXR, as well as ABC transporters like ABCA3 and ABCD1 involved in the bile acid metabolism gene set. These genes have been shown to be associated with cancer chemoresistance in previous studies. EPHX1 is recently reported to induce the resistance to aclarubicin and mitoxantrone in acute myeloid leukemia by regulating several drug-metabolizing enzymes including CYP1A1, glutathione S-transferase Mu 1 (GSTM1), and glutathione S-transferase Theta 1 (GSTT1) (H. Cheng et al., 2019). UGDH is found to be overexpressed in breast cancer patients, being correlated with metastasis and

resultantly worse survival (Teoh, Ogrodzinski, & Lunt, 2020). However, little is known about the role of these two genes in HCC and it is unclear how they could contribute to apatinib resistance in cancer cells. 6PGD, an oxidative carboxylase catalyzing the reduction 6-phosphogluconate into ribulose 5-phosphate in the pentose phosphate pathway, was shown to be up-regulated in HCC and to have critical involvement in the cancer growth and patient survival, where inhibition of 6PGD could sensitize HCC cells to paclitaxel, doxorubicin, and cisplatin (H. Chen et al., 2019). The overexpression of 6PGD may be attributed to the high demand for the release of NADPH from the enzymatic reaction, as NADPH is necessary for fatty acid synthesis and the scavenging of reactive oxygen species that are deregulated in cancer cells.

The association between bile acid metabolism and cancer development as well as drug resistance brings awareness to recent cancer research, as bile acid has long been recognized as an endogenous carcinogen in various types of cancer for more than three decades, and it regulates gene expression during tumor development. Several studies have showed that NEDD4 is involved in growth and metastasis as well as acquisition of EMT in cancers including HCC (Hang et al., 2016; Z. J. Huang, Zhu, Yang, & Biskup, 2017) and cisplatin-resistant nasopharyngeal carcinoma cells (Feng et al., 2017). FXR is a nuclear receptor that can activate the expression of ABCB4 and induce chemoresistance against cisplatin in HCC cells (Vaquero, Briz, Herraez, Muntane, & Marin, 2013), though it remains inconclusive as it is known that FXR also plays a role in maintenance of homeostasis in the liver such as protecting the liver from accumulation of bile acid which can cause damage to the liver. Despite its possible role in regulation acquired drug resistance in some cancers as similar to other ABC transporters (Hu et al., 2018), the FXR-mediated activation of ABCB4 may be

associated with FXR-dependent hepatoprotection against bile acid accumulation, as deficiency of ABCB4 can cause bile regurgitation and damage to the liver (Cariello, Piccinin, Garcia-Irigoyen, Sabba, & Moschetta, 2018).

5.4.2. RhoGDI, SPINK1 and calcium signaling pathways may be the underlying mechanisms of acquired apatinib resistance in HCC cells

The possible pathways involved in regulating the expression and activity of CYP1A1 in PLC/PRF/5 cells are explored by employing the RNA sequencing results in the IPA software. Through the analysis, we discover that lentiviral-based knockdown of CYP1A1 concurrently suppresses the activity of the RhoGDI signaling, SPINK1 pancreatic cancer pathway, and calcium signaling in PLC/PRF/5 cells. Rho GDP dissociation inhibitor (RhoGDI) plays a key role in regulating a vast number of cellular functions through interaction with Rho family GTPases such as Rac1, Cdc42, and RhoA. RhoGDI is identified as a negative regulator of Rho GTPases by preventing nucleotide exchange and their membrane association, but its role in cancer remains to be elucidated. Indeed, RhoGDI is frequently overexpressed in cancers and chemoresistant cancer cell lines (H. J. Cho, Kim, Baek, Kim, & Lee, 2019; Zhang, Zhang, Dagher, & Shacter, 2005), which implies that it may contribute to development of drug resistance in cancer. In HCC, it is found that RhoGDI activates the expression of Rho and hence the PI3K/Akt and MAPK pathway, resulting in metastasis (H. Wang et al., 2014), though the clinical relevance and its role in drug resistance in HCC are lacking. Remarkably, a number of Rho signaling members including Rho guanine nucleotide exchange factors (GEF) such as Ect2, GEF-H1, and MyoGEF, as well as GTPase-activating proteins (GAP) like MgcRacGAP, p190RhoGAP, and MP-GAP are identified as key regulators of mitotic progression, one of which being depleted results

in formation of multinucleated cells. Interestingly, the expression levels of Ect2, GEF-H1, MgcRacGAP, and p190RhoGAP reach a peak at the G2/M boundary (Chircop, 2014). These findings depict that Rho GEF and GAP have their involvement in cell cycle regulation and perhaps some of them may be implicated in G2/M checkpoint regulation. It is also demonstrated that RhoGDIβ regulates mitosis and cytokinesis upon when localized to centrosomes. Dysregulation of RhoGDIβ may contribute to cancer progression by disrupting the regulation of cytokinesis and bipolar spindle formation (Y. S. Jiang et al., 2013). Current knowledge about the causal relationship between RhoGDI signaling and the cell cycle is limited. Our IPA results reveal that RhoGDI signaling is the pathway most down-regulated along with CYP1A1 knockdown. However, it is still unknown whether RhoGDI would mediate apatinib resistance in HCC cells through direct regulation of cell cycle progression and/or interaction with other intracellular signaling pathways. Given the importance of RhoGDI signaling in regulating Rho functions and possibly the cell cycle, future studies may elucidate the key player in RhoGDI signaling that contributes to apatinib resistance.

Serine protease inhibitor Kazal-type 1 (SPINK1) serves to cleave trypsin prematurely activated from trypsinogen stored in the pancreas to prevent the enzyme from causing damage to the organ. Mutation in SPINK1 can cause chronic pancreatitis due to inhibition of its trypsin inactivation function. Up-regulation of SPINK1 is identified to be associated with poor patient survival in multiple cancers including prostate, ovary, bladder, pancreas, colorectal, breast cancers as well as HCC. It is hypothesized that SPINK1 may be secreted by tumor cells to inhibit unknown serine proteases to induce anoikis resistance, tumor cell survival and metastasis, and to activate EGFR kinase pathways leading to tumor cell proliferation (Mehner & Radisky,

2019). Overexpression of SPINK1 has been shown to increase cellular resistance to cell death induced by anti-cancer drugs like 5-fluorouracil, suberoylanilide hydroxamic acid, tamoxifen, adriamycin, and etoposide in estrogen receptor-positive breast cancer cells (Soon et al., 2011). SPINK1 overexpression is also associated with increased risk of carcinogenesis in HBV-related cirrhosis and HCV-related HCC (Hass, Jobst, Vogel, Scheurlen, & Nehls, 2014; F. Li et al., 2015), suggesting that SPINK1 may be utilized by the hepatitis viruses causing the liver diseases. More recent evidence has discovered SPINK1 as a possible downstream target of cadherin-17/β-catenin axis in HCC (Shek et al., 2017). Other studies also document that SPINK1 suppresses E-cadherin and induces metastasis of HCC cells through activation of the MEK/ERK signaling pathway (Ying, Gong, Feng, Jing, & Lu, 2017), notably one of which reveals by GSEA that metabolism of glycine, serine, threonine, and bile acid may be the underlying mechanisms of SPINK1 in HCC (K. Huang et al., 2020), though the detailed roles need further investigation.

Calcium is the most abundant element in the human body and is essential in many physiological functions such as signal transduction and the release of neurotransmitters. The functions of liver carried out by hepatocytes are also governed by calcium ions. Although it is controversial whether calcium can promote the development of cancer, calcium signaling is often dysregulated in chronic liver diseases and HCC (Liang et al., 2018; Modica et al., 2019). The contribution of calcium signaling to acquired drug resistance in cancer cells remains largely unknown. A previous study shows that rutaecarpine, a quinazolinocarboline alkaloid, can induce up-regulation of CYP1A1 expression and activity through activation of the AhR signaling and phosphorylation of Ca(2+)/calmodulin (CaM)-dependent protein kinase (CaMK)

in mouse hepatoma Hepa-1c1c7 cells, thus suggesting a possible role of calcium signaling in mediating xenobiotic metabolism in mammalians (Han, Kim, Im, Jeong, & Jeong, 2009). Another study demonstrates that calpain, a calcium-dependent cysteine protease activated by TCDD-induced intracellular calcium elevation, plays a part in the ligand-dependent transformation and signaling of AhR pathway in Hepa-1c1c7 cells (Dale & Eltom, 2006). More recent evidence also shows that endosulfan, a cyclodiene insecticide, induces CYP1A1 up-regulation through enhanced phosphorylation of CaMK and protein kinase C (PKC) in AhR-expressing cells but not in AhR-deficient mutant cells, emphasizing on the view that calcium signaling is, at least in part, involved in metabolism of xenobiotics (Han, Kim, Lee, & Jeong, 2015). However, whether calcium signaling is a driver inducing xenobiotic-metabolizing enzymes such as CYP1A1 in humans still remains inconclusive, as these studies are all performed on murine cells. Indeed, protein members involved in calcium signaling have a regulatory role in cell cycle progression (Kahl & Means, 2003; Whitaker & Patel, 1990). It has been reported that CaMKII promotes cell cycle progression by directly activating MEK/ERK, which degrades p27 protein that is responsible for inducing G2/M cell cycle arrest (N. Li, Wang, Wu, Liu, & Cao, 2009). This may provide preliminary evidence for the functional connections among CYP1A1, calcium signaling, and G2/M cell cycle checkpoints. Further investigation is required to clarity the role of calcium signaling in xenobiotic metabolism and may help us understand the underlying mechanisms of acquired apatinib resistance in HCC.

CHAPTER 6

CONCLUSIONS AND FUTURE PLAN

Section 6.1 Conclusions

HCC remains one of the deadliest diseases, being the 6th most commonly diagnosed cancer and the 4th leading cause of cancer mortality worldwide. It is prevalent in East Asia and sub-Saharan Africa, which accounts for more than 80% of liver cancer cases in the world. HCC is a complex disease associated with a number of risk factors, including chronic liver disease and cirrhosis, chronic infection with HBV and HCV, alcoholic liver disease, and NAFLD/NASH. Sorafenib is the current standard drug treatment for advanced HCC since its approval. However, the limitations of sorafenib treatment due to drug resistance that frequently results in tumor recurrence have largely increased the demand for developing novel drug interventions against the disease.

Apatinib is a novel anti-angiogenic agent targeting VEGFR2, with encouraging preclinical and clinical performance in treatments of various types of solid tumor including HCC. It has been approved in China since 2014 for advanced gastric cancer, and more extensive clinical studies have been carried out on other cancer types afterward. Increasing body of evidence has proved that apatinib has good safety and potent therapeutic effect on patients with advanced HCC, collectively proposing apatinib as a possible treatment for the disease. However, little is known about the development of acquired resistance to apatinib in HCC. It is of importance to have better understanding about the molecular mechanisms of acquired apatinib resistance to evaluate the therapeutic potential of the drug.

In this study, we first established apatinib-resistant cell lines from two human HCC cell lines PLC/PRF/5 and MHCC-97L by continuous treatment of the cells with

gradually increasing apatinib concentration. This approach is to mimic the clinical conditions of the HCC patients. We characterized the resistant cells by performing a series of functional assays, including apoptosis assay, Western blotting, and migration and invasion assay. In order to uncover the mechanisms underlying apatinib resistance in HCC, we performed RNA sequencing using the mock and apatinib-resistant HCC cells, and through pathway analysis we identified CYP1A1 as the key player whose expression level is significantly up-regulated in the resistant cells.

CYP1A1 has been reported to play a role in cancer. It is involved in metabolism of numerous xenobiotics and endogenous molecules, which gives rise to the hypothesis that the overexpression of CYP1A1 may promote the development of cancer. Early studies show that CYP1A1 polymorphism may have a close association with the risks of various cancer types including HCC. More recent evidence reveals oncogenic contributions by CYP1A1 including enhancement of drug resistance in some cancers. Our study presents an overexpression of CYP1A1 in apatinib-resistant HCC cells, demonstrates the sensitization of apatinib in HCC cells by suppressing the expression of CYP1A1 and its enzymatic activity using lentiviral-based approach, leading to the enhancement of apatinib-induced apoptosis. Collectively, our results are in agreement with the previous findings.

To elucidate the possible molecular mechanisms of CYP1A1 in mediating apatinib resistance in HCC, we analyzed the change in transcriptome pattern in the apatinib-resistant HCC cells and their mock counterparts by performing RNA sequencing and the GSEA. We discover that E2F targets, G2M checkpoint proteins, and MYC targets may support the development of acquired apatinib resistance in HCC

cells. Subsequently, the alternation in transcriptome and signaling pathway profile in HCC cells in response to the CYP1A1 knockdown was explored. We discover that shRNA based CYP1A1 knockdown suppresses xenobiotic and bile acid metabolism in PLC/PRF/5 cells. Meanwhile, our findings provide preliminary evidence showing that RhoGDI signaling, SPINK1 pancreatic cancer pathway, and calcium signaling may be the possible underlying mechanisms of acquired apatinib resistance in the cells.

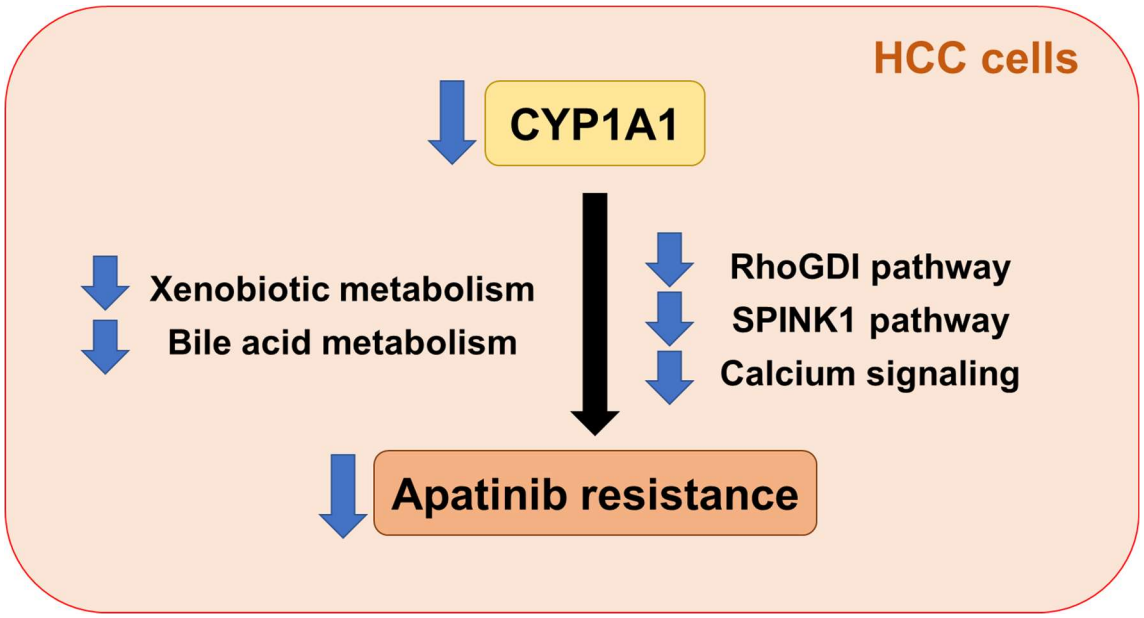

Figure 21. Suppression of CYP1A1 sensitized human HCC cells to apatinib treatment through multiple signaling pathways. Our study discovered that CYP1A1 was highly up-regulated in apatinib-resistant human HCC cells, which contributed to reduction in apoptosis induced by apatinib. Suppression of CYP1A1 reduced apatinib resistance in HCC cells possibly through inhibition of xenobiotic and bile acid metabolism, RhoGDI, SPINK1 and/or calcium signaling pathways.

Section 6.2 Future Plan

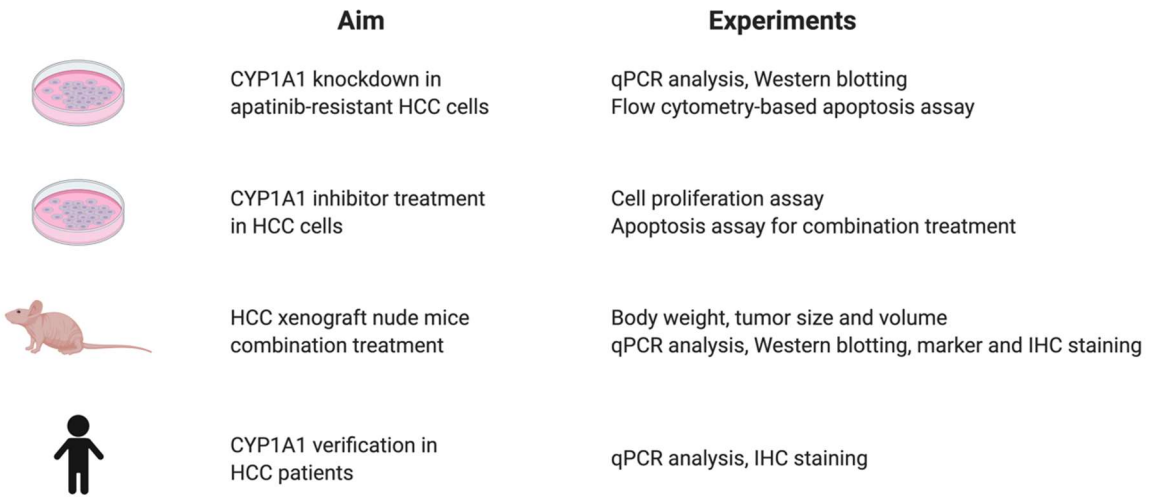

Figure 22. Summary of future plan with aims and experiments.

6.2.1. Examination of the sensitivity of CYP1A1 suppression to apatinib treatment in apatinib-resistant HCC cells

We have shown that knockdown of the CYP1A1 promotes apatinib-induced apoptosis in the mock HCC cells. Our data suggest a potential role of the CYP1A1 in regulating apoptosis in apatinib-treated HCC cells. We therefore expect that the CYP1A1 knockdown would have similar apoptosis-enhancing effects in the apatinib-resistant HCC cells, though we have not testified it in this study. In order to testify the hypothesis that suppression of the CYP1A1 could sensitize apatinib-resistant HCC cells to apatinib treatment, CYP1A1 knockdown clones will be generated from the apatinib-resistant HCC cell lines established, followed by qPCR analysis and Western blotting for verifying the knockdown efficiencies. Afterward, flow cytometry-based apoptosis assay will be performed to compare the apoptosis rates among the knockdown clones and the NTC in response to different apatinib concentrations, as demonstrated in the

previous experiments. The enhancement of apoptosis in the CYP1A1 knockdown clones compared with the NTC will prove the apatinib sensitization in the resistant cells.

6.2.2. Evaluation of the therapeutic effect of α-naphthoflavone, a CYP1A1 inhibitor in HCC cells

Our principal aim is to propose a combination regime for apatinib treatment in order to enhance its therapeutic potential in HCC therapy. In this study, we have identified the CYP1A1 as a potential key player in mediating apatinib resistance in HCC cells. Future experiments will focus on the question whether inhibitors of the CYP1A1 would synchronize with apatinib to reverse the resistance in HCC **(Figure 22)**. α-Naphthoflavone (ANF) is a classical CYP1A1 inhibitor by forming complexes with cytosolic AhR to prevent it from ligand-dependent activation and transformation, suppressing the gene expression of CYP1A1. This compound has been studied in early pharmaceutical studies showing its potent inhibitory effects at concentrations from 10 to 1000 nM (Merchant, Arellano, & Safe, 1990; Santostefano et al., 1993). The therapeutic effect of ANF in HCC cells will be evaluated. Cell proliferation assay such as the MTT assay will be carried out to determine the IC_{50} values of the compound. Subsequent experiments involving the apoptosis assay will examine the combination effect of ANF with apatinib in the parental and apatinib-resistant HCC cells. The hypothesis can be testified *in vitro* by showing the enhancement of apoptosis in the combination groups.

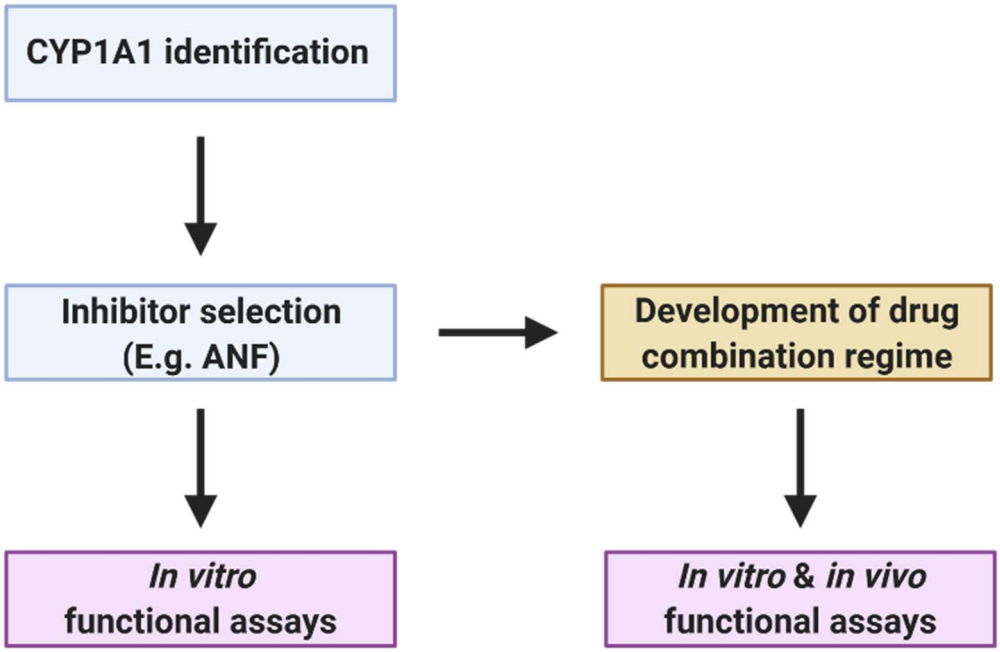

Figure 23. Flow chart of future plan to develop a combination regimen with apatinib.

6.2.3. Evaluation of *in vivo* therapeutic effect of ANF in combination with apatinib in HCC xenograft nude mice model

After demonstrating the combination treatment in HCC cells, extensive experiments will be conducted to testify the regime *in vivo* using HCC xenograft nude mice model. In the experiment, the nude mice subcutaneously injected with the apatinib-resistant HCC cells will be allowed for tumor formation in the liver, and then will be evenly divided into four groups labeled as apatinib only, ANF only, combination, and control, respectively **(Figure 23)**. Each group will be given the corresponding treatment strategy with care for at least eight weeks. Body weight and tumor size will be measured regularly during the treatment. All the mice will be sacrificed afterward to harvest their tumor-bearing liver for further investigation such as qPCR analysis, Western blotting, marker staining, and immunohistochemistry (IHC) staining.

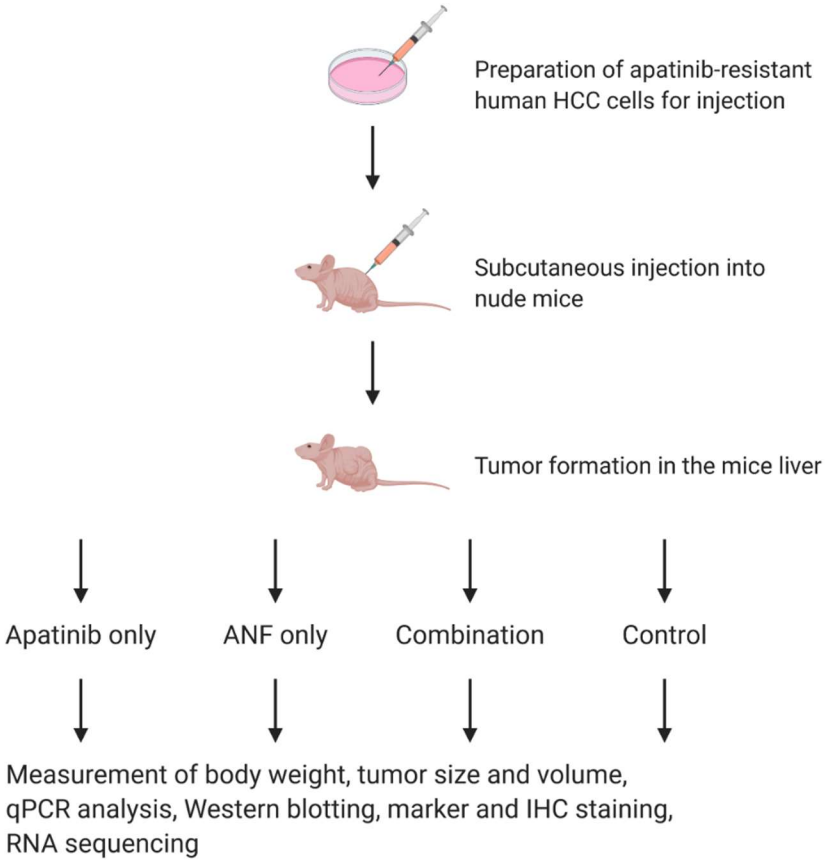

Figure 24. Evaluation of *in vivo* therapeutic effect of ANF in combination with apatinib in HCC xenograft nude mice model.

6.2.4. Further confirmation of the pathways identified by RNA sequencing

In the previous chapter, we have identified several pathways which was found to be significantly downregulated including xenobiotic and bile acid metabolism, RhoGDI, SPINK1 and calcium signaling pathways in response to CYP1A1 knockdown. However, we have not confirmed these pathways in this study. For this purpose, we will compare the expression of the genes that are crucial for these pathways between shCYP1A1 cells and their non-target counterparts by qPCR and western blot analyses.

6.2.5. Verification of CYP1A1 expression in apatinib-treated HCC patients

In collaboration with Dr. Zhu in Shunde Hospital of Southern Medical University, we have obtained HCC samples pre- and post-apatinib treatment. We will correlate with the expression of the CYP1A1 and HCC patients' survival by qPCR analysis and IHC staining. Resultantly, we can prove whether elevation of CYP1A1 upon apatinib treatment is clinically relevant.

List of References

Abou-Alfa, G. K., Meyer, T., Cheng, A. L., El-Khoueiry, A. B., Rimassa, L., Ryoo, B. Y., . . . Kelley, R. K. (2018). Cabozantinib in Patients with Advanced and Progressing Hepatocellular Carcinoma. *N Engl J Med, 379*(1), 54-63. doi:10.1056/NEJMoa1717002

Aimova, D., Svobodova, L., Kotrbova, V., Mrazova, B., Hodek, P., Hudecek, J., . . . Stiborova, M. (2007). The anticancer drug ellipticine is a potent inducer of rat cytochromes P450 1A1 and 1A2, thereby modulating its own metabolism. *Drug Metab Dispos, 35*(10), 1926-1934. doi:10.1124/dmd.107.016048

Al-Dhfyan, A., Alhoshani, A., & Korashy, H. M. (2017). Aryl hydrocarbon receptor/cytochrome P450 1A1 pathway mediates breast cancer stem cells expansion through PTEN inhibition and beta-Catenin and Akt activation. *Mol Cancer, 16*(1), 14. doi:10.1186/s12943-016-0570-y

Alfieri, R. R., Galetti, M., Tramonti, S., Andreoli, R., Mozzoni, P., Cavazzoni, A., . . . Petronini, P. G. (2011). Metabolism of the EGFR tyrosin kinase inhibitor gefitinib by cytochrome P450 1A1 enzyme in EGFR-wild type non small cell lung cancer cell lines. *Mol Cancer, 10*, 143. doi:10.1186/1476-4598-10-143

Andersson, P., McGuire, J., Rubio, C., Gradin, K., Whitelaw, M. L., Pettersson, S., . . . Poellinger, L. (2002). A constitutively active dioxin/aryl hydrocarbon receptor induces stomach tumors. *Proc Natl Acad Sci U S A, 99*(15), 9990-9995. doi:10.1073/pnas.152706299

Androutsopoulos, V. P., Tsatsakis, A. M., & Spandidos, D. A. (2009). Cytochrome P450 CYP1A1: wider roles in cancer progression and prevention. *BMC Cancer, 9*, 187. doi:10.1186/1471-2407-9-187

Axley, P., Ahmed, Z., Ravi, S., & Singal, A. K. (2018). Hepatitis C Virus and Hepatocellular Carcinoma: A Narrative Review. *J Clin Transl Hepatol, 6*(1), 79-84. doi:10.14218/JCTH.2017.00067

Aziz, M. A., Serya, R. A., Lasheen, D. S., Abdel-Aziz, A. K., Esmat, A., Mansour, A. M., . . . Abouzid, K. A. (2016). Discovery of Potent VEGFR-2 Inhibitors based on Furopyrimidine and Thienopyrimidne Scaffolds as Cancer Targeting Agents. *Sci Rep, 6*, 24460. doi:10.1038/srep24460

Backlund, M., Johansson, I., Mkrtchian, S., & Ingelman-Sundberg, M. (1997). Signal transduction-mediated activation of the aryl hydrocarbon receptor in rat hepatoma H4IIE cells. *J Biol Chem, 272*(50), 31755-31763. doi:10.1074/jbc.272.50.31755

Bruix, J., Qin, S., Merle, P., Granito, A., Huang, Y. H., Bodoky, G., . . . Investigators, R. (2017). Regorafenib for patients with hepatocellular carcinoma who progressed on sorafenib treatment (RESORCE): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet, 389*(10064), 56-66. doi:10.1016/S0140-6736(16)32453-9

Bruix, J., Sherman, M., & American Association for the Study of Liver, D. (2011). Management of hepatocellular carcinoma: an update. *Hepatology, 53*(3), 1020-1022. doi:10.1002/hep.24199

Cariello, M., Piccinin, E., Garcia-Irigoyen, O., Sabba, C., & Moschetta, A. (2018). Nuclear receptor FXR, bile acids and liver damage: Introducing the progressive familial intrahepatic cholestasis with FXR mutations. *Biochim Biophys Acta Mol Basis Dis, 1864*(4 Pt B), 1308-1318. doi:10.1016/j.bbadis.2017.09.019

Chen, H., Wu, D., Bao, L., Yin, T., Lei, D., Yu, J., & Tong, X. (2019). 6PGD inhibition sensitizes hepatocellular carcinoma to chemotherapy via AMPK activation and

metabolic reprogramming. *Biomed Pharmacother, 111*, 1353-1358. doi:10.1016/j.biopha.2019.01.028

Chen, S., Yu, W., Zhang, K., & Liu, W. (2018). Comparison of the efficacy and safety of Transarterial chemoembolization with and without Apatinib for the treatment of BCLC stage C hepatocellular carcinoma. *BMC Cancer, 18*(1), 1131. doi:10.1186/s12885-018-5081-3

Chen, X., Qiu, T., Zhu, Y., Sun, J., Li, P., Wang, B., . . . Gu, Y. (2019). A Single-Arm, Phase II Study of Apatinib in Refractory Metastatic Colorectal Cancer. *Oncologist, 24*(7), 883-e407. doi:10.1634/theoncologist.2019-0164

Cheng, H., Huang, C., Tang, G., Qiu, H., Gao, L., Zhang, W., . . . Chen, L. (2019). Emerging role of EPHX1 in chemoresistance of acute myeloid leukemia by regurlating drug-metabolizing enzymes and apoptotic signaling. *Mol Carcinog, 58*(5), 808-819. doi:10.1002/mc.22973

Cheng, Y., Jin, U. H., Allred, C. D., Jayaraman, A., Chapkin, R. S., & Safe, S. (2015). Aryl Hydrocarbon Receptor Activity of Tryptophan Metabolites in Young Adult Mouse Colonocytes. *Drug Metab Dispos, 43*(10), 1536-1543. doi:10.1124/dmd.115.063677

Chircop, M. (2014). Rho GTPases as regulators of mitosis and cytokinesis in mammalian cells. *Small GTPases, 5*. doi:10.4161/sgtp.29770

Cho, H. J., Kim, J. T., Baek, K. E., Kim, B. Y., & Lee, H. G. (2019). Regulation of Rho GTPases by RhoGDIs in Human Cancers. *Cells, 8*(9). doi:10.3390/cells8091037

Cho, I. J., & Kim, S. G. (2003). Oltipraz inhibits 3-methylcholanthrene induction of CYP1A1 by CCAAT/enhancer-binding protein activation. *J Biol Chem, 278*(45), 44103-44112. doi:10.1074/jbc.M307597200

Choi, Y. M., An, S., Lee, E. M., Kim, K., Choi, S. J., Kim, J. S., . . . Bae, S. (2012). CYP1A1 is a target of miR-892a-mediated post-transcriptional repression. *Int J Oncol, 41*(1), 331-336. doi:10.3892/ijo.2012.1418

Cuitino, M. C., Pecot, T., Sun, D., Kladney, R., Okano-Uchida, T., Shinde, N., . . . Leone, G. (2019). Two Distinct E2F Transcriptional Modules Drive Cell Cycles and Differentiation. *Cell Rep, 27*(12), 3547-3560 e3545. doi:10.1016/j.celrep.2019.05.004

Dale, Y. R., & Eltom, S. E. (2006). Calpain mediates the dioxin-induced activation and down-regulation of the aryl hydrocarbon receptor. *Mol Pharmacol, 70*(5), 1481-1487. doi:10.1124/mol.106.027474

Dvorak, Z., Ulrichova, J., & Modriansky, M. (2005). Role of microtubules network in CYP genes expression. *Curr Drug Metab, 6*(6), 545-552. doi:10.2174/138920005774832623

Dvorak, Z., Vrzal, R., Ulrichova, J., Pascussi, J. M., Maurel, P., & Modriansky, M. (2006). Involvement of cytoskeleton in AhR-dependent CYP1A1 expression. *Curr Drug Metab, 7*(3), 301-313. doi:10.2174/138920006776359310

El-Khoueiry, A. B., Sangro, B., Yau, T., Crocenzi, T. S., Kudo, M., Hsu, C., . . . Melero, I. (2017). Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. *Lancet, 389*(10088), 2492-2502. doi:10.1016/S0140-6736(17)31046-2

Elmeligie, S., Aboul-Magd, A. M., Lasheen, D. S., Ibrahim, T. M., Abdelghany, T. M., Khojah, S. M., & Abouzid, K. A. M. (2019). Design and synthesis of phthalazine-based compounds as potent anticancer agents with potential antiangiogenic activity via VEGFR-2 inhibition. *J Enzyme Inhib Med Chem, 34*(1), 1347-1367. doi:10.1080/14756366.2019.1642883

Ezzeldin, N., El-Lebedy, D., Darwish, A., El-Bastawisy, A., Hassan, M., Abd El-Aziz, S., . . . Saad-Hussein, A. (2017). Genetic polymorphisms of human cytochrome P450 CYP1A1 in an Egyptian population and tobacco-induced lung cancer. *Genes Environ, 39*, 7. doi:10.1186/s41021-016-0066-4

Fallone, F., Villard, P. H., Seree, E., Rimet, O., Nguyen, Q. B., Bourgarel-Rey, V., . . . Lacarelle, B. (2004). Retinoids repress Ah receptor CYP1A1 induction pathway through the SMRT corepressor. *Biochem Biophys Res Commun, 322*(2), 551-556. doi:10.1016/j.bbrc.2004.07.153

Feng, S., Yang, G., Yang, H., Liang, Z., Zhang, R., Fan, Y., & Zhang, G. (2017). NEDD4 is involved in acquisition of epithelial-mesenchymal transition in cisplatin-resistant nasopharyngeal carcinoma cells. *Cell Cycle, 16*(9), 869-878. doi:10.1080/15384101.2017.1308617

Fleming, F. F., Yao, L., Ravikumar, P. C., Funk, L., & Shook, B. C. (2010). Nitrile-containing pharmaceuticals: efficacious roles of the nitrile pharmacophore. *J Med Chem, 53*(22), 7902-7917. doi:10.1021/jm100762r

Furet, P., Bold, G., Hofmann, F., Manley, P., Meyer, T., & Altmann, K. H. (2003). Identification of a new chemical class of potent angiogenesis inhibitors based on conformational considerations and database searching. *Bioorg Med Chem Lett, 13*(18), 2967-2971. doi:10.1016/s0960-894x(03)00626-7

Giangrande, P. H., Zhu, W., Schlisio, S., Sun, X., Mori, S., Gaubatz, S., & Nevins, J. R. (2004). A role for E2F6 in distinguishing G1/S- and G2/M-specific transcription. *Genes Dev, 18*(23), 2941-2951. doi:10.1101/gad.1239304

Hamouchene, H., Arlt, V. M., Giddings, I., & Phillips, D. H. (2011). Influence of cell cycle on responses of MCF-7 cells to benzo[a]pyrene. *BMC Genomics, 12*, 333. doi:10.1186/1471-2164-12-333

Han, E. H., Kim, H. G., Im, J. H., Jeong, T. C., & Jeong, H. G. (2009). Up-regulation of CYP1A1 by rutaecarpine is dependent on aryl hydrocarbon receptor and calcium. *Toxicology, 266*(1-3), 38-47. doi:10.1016/j.tox.2009.10.013

Han, E. H., Kim, H. G., Lee, E. J., & Jeong, H. G. (2015). Endosulfan Induces CYP1A1 Expression Mediated through Aryl Hydrocarbon Receptor Signal Transduction by Protein Kinase C. *Toxicol Res, 31*(4), 339-345. doi:10.5487/TR.2015.31.4.339

Hang, X., Zhu, S., Di, H., Wu, Z., Chu, K., Wang, J., . . . Xu, W. (2016). NEDD4 Depletion Inhibits Hepatocellular Carcinoma Growth via Targeting PTEN. *Cell Physiol Biochem, 39*(2), 768-779. doi:10.1159/000445667

Hass, H. G., Jobst, J., Vogel, U., Scheurlen, M., & Nehls, O. (2014). Overexpression of tumor-associated trypsin inhibitor (SPINK1/TATI) in hepatitis C-associated hepatocellular carcinoma: potential implications for viral hepatocarcinogenesis. *Oncol Res Treat, 37*(12), 732-738. doi:10.1159/000369151

He, X., Huang, Z., Liu, P., Li, Q., Wang, M., Qiu, M., . . . Yang, S. (2020). Apatinib Inhibits the Invasion and Metastasis of Liver Cancer Cells by Downregulating MMP-Related Proteins via Regulation of the NF-kappaB Signaling Pathway. *Biomed Res Int, 2020*, 3126182. doi:10.1155/2020/3126182

Heng, Y. W., & Koh, C. G. (2010). Actin cytoskeleton dynamics and the cell division cycle. *Int J Biochem Cell Biol, 42*(10), 1622-1633. doi:10.1016/j.biocel.2010.04.007

Hirvonen, A., Husgafvel-Pursiainen, K., Karjalainen, A., Anttila, S., & Vainio, H. (1992). Point-mutational MspI and Ile-Val polymorphisms closely linked in the CYP1A1 gene: lack of association with susceptibility to lung cancer in a Finnish study population. *Cancer Epidemiol Biomarkers Prev, 1*(6), 485-489. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/1284589

Hu, H., Wang, M., Guan, X., Yuan, Z., Liu, Z., Zou, C., . . . Wang, X. (2018). Loss of ABCB4 attenuates the caspase-dependent apoptosis regulating resistance to 5-Fu in colorectal cancer. *Biosci Rep, 38*(1). doi:10.1042/BSR20171428

Huang, K., Xie, W., Wang, S., Li, Q., Wei, X., Chen, B., . . . Shen, S. (2020). High SPINK1 Expression Predicts Poor Prognosis and Promotes Cell Proliferation and Metastasis of Hepatocellular Carcinoma. *J Invest Surg*, 1-10. doi:10.1080/08941939.2020.1728443

Huang, Z. J., Zhu, J. J., Yang, X. Y., & Biskup, E. (2017). NEDD4 promotes cell growth and migration via PTEN/PI3K/AKT signaling in hepatocellular carcinoma. *Oncol Lett, 14*(3), 2649-2656. doi:10.3892/ol.2017.6532

Inoue, K., Asao, T., & Shimada, T. (2000). Ethnic-related differences in the frequency distribution of genetic polymorphisms in the CYP1A1 and CYP1B1 genes in Japanese and Caucasian populations. *Xenobiotica, 30*(3), 285-295. doi:10.1080/004982500237677

Jiang, Y. S., Maeda, M., Okamoto, M., Fujii, M., Fukutomi, R., Hori, M., . . . Ota, T. (2013). Centrosomal localization of RhoGDIbeta and its relevance to mitotic processes in cancer cells. *Int J Oncol, 42*(2), 460-468. doi:10.3892/ijo.2012.1730

Jiang, Z., Dalton, T. P., Jin, L., Wang, B., Tsuneoka, Y., Shertzer, H. G., . . . Nebert, D. W. (2005). Toward the evaluation of function in genetic variability: characterizing human SNP frequencies and establishing BAC-transgenic mice carrying the human CYP1A1_CYP1A2 locus. *Hum Mutat, 25*(2), 196-206. doi:10.1002/humu.20134

Jorge-Nebert, L. F., Jiang, Z., Chakraborty, R., Watson, J., Jin, L., McGarvey, S. T., . . . Nebert, D. W. (2010). Analysis of human CYP1A1 and CYP1A2 genes and their shared bidirectional promoter in eight world populations. *Hum Mutat, 31*(1), 27-40. doi:10.1002/humu.21132

Kahl, C. R., & Means, A. R. (2003). Regulation of cell cycle progression by calcium/calmodulin-dependent pathways. *Endocr Rev, 24*(6), 719-736. doi:10.1210/er.2003-0008

Kanda, T., Goto, T., Hirotsu, Y., Moriyama, M., & Omata, M. (2019). Molecular Mechanisms Driving Progression of Liver Cirrhosis towards Hepatocellular Carcinoma in Chronic Hepatitis B and C Infections: A Review. *Int J Mol Sci, 20*(6). doi:10.3390/ijms20061358

Kong, Y., Sun, L., Hou, Z., Zhang, Y., Chen, P., Cui, Y., . . . Qin, L. (2017). Apatinib is effective for treatment of advanced hepatocellular carcinoma. *Oncotarget, 8*(62), 105596-105605. doi:10.18632/oncotarget.22337

Kudo, M., Finn, R. S., Qin, S., Han, K. H., Ikeda, K., Piscaglia, F., . . . Cheng, A. L. (2018). Lenvatinib versus sorafenib in first-line treatment of patients with unresectable hepatocellular carcinoma: a randomised phase 3 non-inferiority trial. *Lancet, 391*(10126), 1163-1173. doi:10.1016/S0140-6736(18)30207-1

Lan, C. Y., Wang, Y., Xiong, Y., Li, J. D., Shen, J. X., Li, Y. F., . . . Huang, X. (2018). Apatinib combined with oral etoposide in patients with platinum-resistant or platinum-refractory ovarian cancer (AEROC): a phase 2, single-arm, prospective study. *Lancet Oncol, 19*(9), 1239-1246. doi:10.1016/S1470-2045(18)30349-8

Lang, D., Radtke, M., & Bairlein, M. (2019). Highly Variable Expression of CYP1A1 in Human Liver and Impact on Pharmacokinetics of Riociguat and Granisetron in Humans. *Chem Res Toxicol, 32*(6), 1115-1122. doi:10.1021/acs.chemrestox.8b00413

Le Devedec, S. E., Yan, K., de Bont, H., Ghotra, V., Truong, H., Danen, E. H., . . . van de Water, B. (2010). Systems microscopy approaches to understand cancer cell migration and metastasis. *Cell Mol Life Sci, 67*(19), 3219-3240. doi:10.1007/s00018-010-0419-2

Lee, A. J., Cai, M. X., Thomas, P. E., Conney, A. H., & Zhu, B. T. (2003). Characterization of the oxidative metabolites of 17beta-estradiol and estrone formed by 15 selectively expressed human cytochrome p450 isoforms. *Endocrinology, 144*(8), 3382-3398. doi:10.1210/en.2003-0192

Lemoine, M., Nayagam, S., & Thursz, M. (2013). Viral hepatitis in resource-limited countries and access to antiviral therapies: current and future challenges. *Future Virol, 8*(4), 371-380. doi:10.2217/fvl.13.11

Li, E., & Hristova, K. (2006). Role of receptor tyrosine kinase transmembrane domains in cell signaling and human pathologies. *Biochemistry, 45*(20), 6241-6251. doi:10.1021/bi060609y

Li, F., Liu, T., Xiao, C. Y., Yu, J. X., Lu, L. G., & Xu, M. Y. (2015). FOXP1 and SPINK1 reflect the risk of cirrhosis progression to HCC with HBV infection. *Biomed Pharmacother, 72*, 103-108. doi:10.1016/j.biopha.2015.04.006

Li, J., Zhao, X., Chen, L., Guo, H., Lv, F., Jia, K., . . . Zuo, Y. (2010). Safety and pharmacokinetics of novel selective vascular endothelial growth factor receptor-2 inhibitor YN968D1 in patients with advanced malignancies. *BMC Cancer, 10*, 529. doi:10.1186/1471-2407-10-529

Li, N., Wang, C., Wu, Y., Liu, X., & Cao, X. (2009). Ca(2+)/calmodulin-dependent protein kinase II promotes cell cycle progression by directly activating MEK1 and subsequently modulating p27 phosphorylation. *J Biol Chem, 284*(5), 3021-3027. doi:10.1074/jbc.M805483200

Li, Q., Qin, S., Gu, S., Chen, X., Lin, L., Wang, Z., . . . Jin, C. (2020). Apatinib as second-line therapy in Chinese patients with advanced hepatocellular carcinoma: A randomized, placebo-controlled, double-blind, phase III study. *Journal of Clinical Oncology, 38*(15_suppl), 4507-4507. doi:10.1200/JCO.2020.38.15_suppl.4507

Li, R., Shugart, Y. Y., Zhou, W., An, Y., Yang, Y., Zhou, Y., . . . Jin, L. (2009). Common genetic variations of the cytochrome P450 1A1 gene and risk of hepatocellular carcinoma in a Chinese population. *Eur J Cancer, 45*(7), 1239-1247. doi:10.1016/j.ejca.2008.11.007

Liang, J. Q., Teoh, N., Xu, L., Pok, S., Li, X., Chu, E. S. H., . . . Yu, J. (2018). Dietary cholesterol promotes steatohepatitis related hepatocellular carcinoma through dysregulated metabolism and calcium signaling. *Nat Commun, 9*(1), 4490. doi:10.1038/s41467-018-06931-6

Lin, Kostov, R., Huang, J. T., Henderson, C. J., & Wolf, C. R. (2017). Novel Pathways of Ponatinib Disposition Catalyzed By CYP1A1 Involving Generation of Potentially Toxic Metabolites. *J Pharmacol Exp Ther, 363*(1), 12-19. doi:10.1124/jpet.117.243246

Liu, P., Xie, S. H., Hu, S., Cheng, X., Gao, T., Zhang, C., & Song, Z. (2017). Age-specific sex difference in the incidence of hepatocellular carcinoma in the United States. *Oncotarget, 8*(40), 68131-68137. doi:10.18632/oncotarget.19245

Llovet, J. M., Bru, C., & Bruix, J. (1999). Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin Liver Dis, 19*(3), 329-338. doi:10.1055/s-2007-1007122

Llovet, J. M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J. F., . . . Group, S. I. S. (2008). Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med, 359*(4), 378-390. doi:10.1056/NEJMoa0708857

Lu, W., Jin, X. L., Yang, C., Du, P., Jiang, F. Q., Ma, J. P., . . . Zhang, Z. (2017). Comparison of efficacy between TACE combined with apatinib and TACE alone in the treatment of intermediate and advanced hepatocellular carcinoma: A single-center randomized controlled trial. *Cancer Biol Ther, 18*(6), 433-438. doi:10.1080/15384047.2017.1323589

Ma, X., Idle, J. R., Krausz, K. W., & Gonzalez, F. J. (2005). Metabolism of melatonin by human cytochromes p450. *Drug Metab Dispos, 33*(4), 489-494. doi:10.1124/dmd.104.002410

MacLeod, A. K., Lin, Huang, J. T., McLaughlin, L. A., Henderson, C. J., & Wolf, C. R. (2018). Identification of Novel Pathways of Osimertinib Disposition and Potential Implications

for the Outcome of Lung Cancer Therapy. *Clin Cancer Res, 24*(9), 2138-2147. doi:10.1158/1078-0432.CCR-17-3555

Manley, P. W., Bold, G., Bruggen, J., Fendrich, G., Furet, P., Mestan, J., . . . Wood, J. (2004). Advances in the structural biology, design and clinical development of VEGF-R kinase inhibitors for the treatment of angiogenesis. *Biochim Biophys Acta, 1697*(1-2), 17-27. doi:10.1016/j.bbapap.2003.11.010

Manley, P. W., Furet, P., Bold, G., Bruggen, J., Mestan, J., Meyer, T., . . . Thierauch, K. H. (2002). Anthranilic acid amides: a novel class of antiangiogenic VEGF receptor kinase inhibitors. *J Med Chem, 45*(26), 5687-5693. doi:10.1021/jm020899q

Manzella, C., Singhal, M., Alrefai, W. A., Saksena, S., Dudeja, P. K., & Gill, R. K. (2018). Serotonin is an endogenous regulator of intestinal CYP1A1 via AhR. *Sci Rep, 8*(1), 6103. doi:10.1038/s41598-018-24213-5

Marumoto, S., Oda, Y., & Miyazawa, M. (2011). Antigenotoxic activity of naturally occurring furanocoumarins. *Environ Mol Mutagen, 52*(8), 646-657. doi:10.1002/em.20665

Matsui, J., Yamamoto, Y., Funahashi, Y., Tsuruoka, A., Watanabe, T., Wakabayashi, T., . . . Asada, M. (2008). E7080, a novel inhibitor that targets multiple kinases, has potent antitumor activities against stem cell factor producing human small cell lung cancer H146, based on angiogenesis inhibition. *Int J Cancer, 122*(3), 664-671. doi:10.1002/ijc.23131

McTigue, M., Murray, B. W., Chen, J. H., Deng, Y. L., Solowiej, J., & Kania, R. S. (2012). Molecular conformations, interactions, and properties associated with drug efficiency and clinical performance among VEGFR TK inhibitors. *Proc Natl Acad Sci U S A, 109*(45), 18281-18289. doi:10.1073/pnas.1207759109

Mehner, C., & Radisky, E. S. (2019). Bad Tumors Made Worse: SPINK1. *Front Cell Dev Biol, 7*, 10. doi:10.3389/fcell.2019.00010

Merchant, M., Arellano, L., & Safe, S. (1990). The mechanism of action of alpha-naphthoflavone as an inhibitor of 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced CYP1A1 gene expression. *Arch Biochem Biophys, 281*(1), 84-89. doi:10.1016/0003-9861(90)90416-v

Miyazaki, M., Sugawara, E., Yoshimura, T., Yamazaki, H., & Kamataki, T. (2005). Mutagenic activation of betel quid-specific N-nitrosamines catalyzed by human cytochrome P450 coexpressed with NADPH-cytochrome P450 reductase in Salmonella typhimurium YG7108. *Mutat Res, 581*(1-2), 165-171. doi:10.1016/j.mrgentox.2004.12.002

Modica, T. M. E., Dituri, F., Mancarella, S., Pisano, C., Fabregat, I., & Giannelli, G. (2019). Calcium Regulates HCC Proliferation as well as EGFR Recycling/Degradation and Could Be a New Therapeutic Target in HCC. *Cancers (Basel), 11*(10). doi:10.3390/cancers11101588

Nakaseko, Y., & Yanagida, M. (2001). Cell biology. Cytoskeleton in the cell cycle. *Nature, 412*(6844), 291-292. doi:10.1038/35085684

Nayagam, S., Thursz, M., Sicuri, E., Conteh, L., Wiktor, S., Low-Beer, D., & Hallett, T. B. (2016). Requirements for global elimination of hepatitis B: a modelling study. *Lancet Infect Dis, 16*(12), 1399-1408. doi:10.1016/S1473-3099(16)30204-3

Nguyen, C. H., Brenner, S., Huttary, N., Atanasov, A. G., Dirsch, V. M., Chatuphonprasert, W., . . . Jager, W. (2016). AHR/CYP1A1 interplay triggers lymphatic barrier breaching in breast cancer spheroids by inducing 12(S)-HETE synthesis. *Hum Mol Genet, 25*(22), 5006-5016. doi:10.1093/hmg/ddw329

Okamoto, K., Ikemori-Kawada, M., Jestel, A., von Konig, K., Funahashi, Y., Matsushima, T., . . . Matsui, J. (2015). Distinct binding mode of multikinase inhibitor lenvatinib revealed by biochemical characterization. *ACS Med Chem Lett, 6*(1), 89-94. doi:10.1021/ml500394m

Otto, T., & Sicinski, P. (2017). Cell cycle proteins as promising targets in cancer therapy. *Nat Rev Cancer, 17*(2), 93-115. doi:10.1038/nrc.2016.138

Park, J. W., Reed, J. R., & Backes, W. L. (2015). The Localization of Cytochrome P450s CYP1A1 and CYP1A2 into Different Lipid Microdomains Is Governed by Their N-terminal and Internal Protein Regions. *J Biol Chem, 290*(49), 29449-29460. doi:10.1074/jbc.M115.687103

Park, J. W., Reed, J. R., Brignac-Huber, L. M., & Backes, W. L. (2014). Cytochrome P450 system proteins reside in different regions of the endoplasmic reticulum. *Biochem J, 464*(2), 241-249. doi:10.1042/BJ20140787

Petrick, J. L., Campbell, P. T., Koshiol, J., Thistle, J. E., Andreotti, G., Beane-Freeman, L. E., . . . McGlynn, K. A. (2018). Tobacco, alcohol use and risk of hepatocellular carcinoma and intrahepatic cholangiocarcinoma: The Liver Cancer Pooling Project. *Br J Cancer, 118*(7), 1005-1012. doi:10.1038/s41416-018-0007-z

Qin, S. (2014). Apatinib in Chinese patients with advanced hepatocellular carcinoma: A phase II randomized, open-label trial. *Journal of Clinical Oncology, 32*(15_suppl), 4019-4019. doi:10.1200/jco.2014.32.15_suppl.4019

Ramakrishna, G., Rastogi, A., Trehanpati, N., Sen, B., Khosla, R., & Sarin, S. K. (2013). From cirrhosis to hepatocellular carcinoma: new molecular insights on inflammation and cellular senescence. *Liver Cancer, 2*(3-4), 367-383. doi:10.1159/000343852

Ren, B., Cam, H., Takahashi, Y., Volkert, T., Terragni, J., Young, R. A., & Dynlacht, B. D. (2002). E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. *Genes Dev, 16*(2), 245-256. doi:10.1101/gad.949802

Rochat, B., Zoete, V., Grosdidier, A., von Grunigen, S., Marull, M., & Michielin, O. (2008). In vitro biotransformation of imatinib by the tumor expressed CYP1A1 and CYP1B1. *Biopharm Drug Dispos, 29*(2), 103-118. doi:10.1002/bdd.598

Rodriguez, M., & Potter, D. A. (2013). CYP1A1 regulates breast cancer proliferation and survival. *Mol Cancer Res, 11*(7), 780-792. doi:10.1158/1541-7786.MCR-12-0675

Santini, R. P., Myrand, S., Elferink, C., & Reiners, J. J., Jr. (2001). Regulation of Cyp1a1 induction by dioxin as a function of cell cycle phase. *J Pharmacol Exp Ther, 299*(2), 718-728. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11602686

Santostefano, M., Merchant, M., Arellano, L., Morrison, V., Denison, M. S., & Safe, S. (1993). alpha-Naphthoflavone-induced CYP1A1 gene expression and cytosolic aryl hydrocarbon receptor transformation. *Mol Pharmacol, 43*(2), 200-206. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/8381508

Sanyal, A. J., Yoon, S. K., & Lencioni, R. (2010). The etiology of hepatocellular carcinoma and consequences for treatment. *Oncologist, 15 Suppl 4*, 14-22. doi:10.1634/theoncologist.2010-S4-14

Schiering, C., Wincent, E., Metidji, A., Iseppon, A., Li, Y., Potocnik, A. J., . . . Stockinger, B. (2017). Feedback control of AHR signalling regulates intestinal immunity. *Nature, 542*(7640), 242-245. doi:10.1038/nature21080

Scholler, A., Hong, N. J., Bischer, P., & Reiners, J. J., Jr. (1994). Short and long term effects of cytoskeleton-disrupting drugs on cytochrome P450 Cyp1a-1 induction in murine hepatoma 1c1c7 cells: suppression by the microtubule inhibitor nocodazole. *Mol Pharmacol, 45*(5), 944-954. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/8190110

Schulthess, P., Loffler, A., Vetter, S., Kreft, L., Schwarz, M., Braeuning, A., & Bluthgen, N. (2015). Signal integration by the CYP1A1 promoter--a quantitative study. *Nucleic Acids Res, 43*(11), 5318-5330. doi:10.1093/nar/gkv423

Schwarz, D., Kisselev, P., Ericksen, S. S., Szklarz, G. D., Chernogolov, A., Honeck, H., . . . Roots, I. (2004). Arachidonic and eicosapentaenoic acid metabolism by human

CYP1A1: highly stereoselective formation of 17(R),18(S)-epoxyeicosatetraenoic acid. *Biochem Pharmacol, 67*(8), 1445-1457. doi:10.1016/j.bcp.2003.12.023

Scott, E. E., Wolf, C. R., Otyepka, M., Humphreys, S. C., Reed, J. R., Henderson, C. J., . . . Backes, W. L. (2016). The Role of Protein-Protein and Protein-Membrane Interactions on P450 Function. *Drug Metab Dispos, 44*(4), 576-590. doi:10.1124/dmd.115.068569

Seaton, M. J., Schlosser, P. M., Bond, J. A., & Medinsky, M. A. (1994). Benzene metabolism by human liver microsomes in relation to cytochrome P450 2E1 activity. *Carcinogenesis, 15*(9), 1799-1806. doi:10.1093/carcin/15.9.1799

Shah, M. A., & Schwartz, G. K. (2001). Cell cycle-mediated drug resistance: an emerging concept in cancer therapy. *Clin Cancer Res, 7*(8), 2168-2181. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11489790

Shek, F. H., Luo, R., Lam, B. Y. H., Sung, W. K., Lam, T. W., Luk, J. M., . . . Lee, N. P. (2017). Serine peptidase inhibitor Kazal type 1 (SPINK1) as novel downstream effector of the cadherin-17/beta-catenin axis in hepatocellular carcinoma. *Cell Oncol (Dordr), 40*(5), 443-456. doi:10.1007/s13402-017-0332-x

Shimada, T., Martin, M. V., Pruess-Schwartz, D., Marnett, L. J., & Guengerich, F. P. (1989). Roles of individual human cytochrome P-450 enzymes in the bioactivation of benzo(a)pyrene, 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons. *Cancer Res, 49*(22), 6304-6312. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/2509067

Shimada, T., Oda, Y., Gillam, E. M., Guengerich, F. P., & Inoue, K. (2001). Metabolic activation of polycyclic aromatic hydrocarbons and other procarcinogens by cytochromes P450 1A1 and P450 1B1 allelic variants and other human cytochromes P450 in Salmonella typhimurium NM2009. *Drug Metab Dispos, 29*(9), 1176-1182. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11502724

Silva, I. D., Rodrigues, A. S., Gaspar, J., Maia, R., Laires, A., & Rueff, J. (1997). Involvement of rat cytochrome 1A1 in the biotransformation of kaempferol to quercetin: relevance to the genotoxicity of kaempferol. *Mutagenesis, 12*(5), 383-390. doi:10.1093/mutage/12.5.383

Soon, W. W., Miller, L. D., Black, M. A., Dalmasso, C., Chan, X. B., Pang, B., . . . Liu, E. T. (2011). Combined genomic and phenotype screening reveals secretory factor SPINK1 as an invasion and survival factor associated with patient prognosis in breast cancer. *EMBO Mol Med, 3*(8), 451-464. doi:10.1002/emmm.201100150

Stiborova, M., Hajek, M., Frei, E., & Schmeiser, H. H. (2001). Carcinogenic and nephrotoxic alkaloids aristolochic acids upon activation by NADPH : cytochrome P450 reductase form adducts found in DNA of patients with Chinese herbs nephropathy. *Gen Physiol Biophys, 20*(4), 375-392. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11989648

Stickel, F., Datz, C., Hampe, J., & Bataller, R. (2017). Pathophysiology and Management of Alcoholic Liver Disease: Update 2016. *Gut Liver, 11*(2), 173-188. doi:10.5009/gnl16477

Teng, F., Xu, Z., Chen, J., Zheng, G., Zheng, G., Lv, H., . . . Cheng, X. (2018). DUSP1 induces apatinib resistance by activating the MAPK pathway in gastric cancer. *Oncol Rep, 40*(3), 1203-1222. doi:10.3892/or.2018.6520

Teng, F., Xu, Z. Y., Lyu, H., Wang, Y. P., Wang, L. J., Huang, T., . . . Cheng, X. D. (2018). [Triptolide reverses apatinib resistance in gastric cancer cell line MKN45 via inhibition of heat shock protein 70]. *Zhonghua Zhong Liu Za Zhi, 40*(2), 92-98. doi:10.3760/cma.j.issn.0253-3766.2018.02.003

Teoh, S. T., Ogrodzinski, M. P., & Lunt, S. Y. (2020). UDP-glucose 6-dehydrogenase knockout impairs migration and decreases in vivo metastatic ability of breast cancer cells. *Cancer Letters*. doi:https://doi.org/10.1016/j.canlet.2020.07.031

Terashima, J., Tachikawa, C., Kudo, K., Habano, W., & Ozawa, S. (2013). An aryl hydrocarbon receptor induces VEGF expression through ATF4 under glucose deprivation in HepG2. *BMC Mol Biol, 14*, 27. doi:10.1186/1471-2199-14-27

Testino, G., Leone, S., & Borro, P. (2014). Alcohol and hepatocellular carcinoma: a review and a point of view. *World J Gastroenterol, 20*(43), 15943-15954. doi:10.3748/wjg.v20.i43.15943

Tian, S., Quan, H., Xie, C., Guo, H., Lu, F., Xu, Y., . . . Lou, L. (2011). YN968D1 is a novel and selective inhibitor of vascular endothelial growth factor receptor-2 tyrosine kinase with potent activity in vitro and in vivo. *Cancer Sci, 102*(7), 1374-1380. doi:10.1111/j.1349-7006.2011.01939.x

Tutusaus, A., Stefanovic, M., Boix, L., Cucarull, B., Zamora, A., Blasco, L., . . . Morales, A. (2018). Antiapoptotic BCL-2 proteins determine sorafenib/regorafenib resistance and BH3-mimetic efficacy in hepatocellular carcinoma. *Oncotarget, 9*(24), 16701-16717. doi:10.18632/oncotarget.24673

Valastyan, S., & Weinberg, R. A. (2011). Tumor metastasis: molecular insights and evolving paradigms. *Cell, 147*(2), 275-292. doi:10.1016/j.cell.2011.09.024

Vaquero, J., Briz, O., Herraez, E., Muntane, J., & Marin, J. J. (2013). Activation of the nuclear receptor FXR enhances hepatocyte chemoprotection and liver tumor chemoresistance against genotoxic compounds. *Biochim Biophys Acta, 1833*(10), 2212-2219. doi:10.1016/j.bbamcr.2013.05.006

Vecchini, F., Lenoir-Viale, M. C., Cathelineau, C., Magdalou, J., Bernard, B. A., & Shroot, B. (1994). Presence of a retinoid responsive element in the promoter region of the human cytochrome P4501A1 gene. *Biochem Biophys Res Commun, 201*(3), 1205-1212. doi:10.1006/bbrc.1994.1833

Veldhoen, M., Hirota, K., Westendorf, A. M., Buer, J., Dumoutier, L., Renauld, J. C., & Stockinger, B. (2008). The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature, 453*(7191), 106-109. doi:10.1038/nature06881

Vignjevic, D., & Montagnac, G. (2008). Reorganisation of the dendritic actin network during cancer cell migration and invasion. *Semin Cancer Biol, 18*(1), 12-22. doi:10.1016/j.semcancer.2007.08.001

Wang, H., Wang, B., Liao, Q., An, H., Li, W., Jin, X., . . . Zhao, L. (2014). Overexpression of RhoGDI, a novel predictor of distant metastasis, promotes cell proliferation and migration in hepatocellular carcinoma. *FEBS Lett, 588*(3), 503-508. doi:10.1016/j.febslet.2013.12.016

Wang, Y., Peng, C., Wang, G., Xu, Z., Luo, Y., Wang, J., & Zhu, W. (2019). Exploring binding mechanisms of VEGFR2 with three drugs lenvatinib, sorafenib, and sunitinib by molecular dynamics simulation and free energy calculation. *Chem Biol Drug Des, 93*(5), 934-948. doi:10.1111/cbdd.13493

Wei, Y. D., Bergander, L., Rannug, U., & Rannug, A. (2000). Regulation of CYP1A1 transcription via the metabolism of the tryptophan-derived 6-formylindolo[3,2-b]carbazole. *Arch Biochem Biophys, 383*(1), 99-107. doi:10.1006/abbi.2000.2037

Whitaker, M., & Patel, R. (1990). Calcium and cell cycle control. *Development, 108*(4), 525-542. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/2167196

Wilhelm, S. M., Carter, C., Tang, L., Wilkie, D., McNabola, A., Rong, H., . . . Trail, P. A. (2004). BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression

and angiogenesis. *Cancer Res, 64*(19), 7099-7109. doi:10.1158/0008-5472.CAN-04-1443

Wilhelm, S. M., Dumas, J., Adnane, L., Lynch, M., Carter, C. A., Schutz, G., . . . Zopf, D. (2011). Regorafenib (BAY 73-4506): a new oral multikinase inhibitor of angiogenic, stromal and oncogenic receptor tyrosine kinases with potent preclinical antitumor activity. *Int J Cancer, 129*(1), 245-255. doi:10.1002/ijc.25864

Willis, A. J., Indra, R., Wohak, L. E., Sozeri, O., Feser, K., Mrizova, I., . . . Arlt, V. M. (2018). The impact of chemotherapeutic drugs on the CYP1A1-catalysed metabolism of the environmental carcinogen benzo[a]pyrene: Effects in human colorectal HCT116 TP53(+/+), TP53(+/-) and TP53(-/-) cells. *Toxicology, 398-399*, 1-12. doi:10.1016/j.tox.2018.02.006

Wood, J. M., Bold, G., Buchdunger, E., Cozens, R., Ferrari, S., Frei, J., . . . Totzke, F. (2000). PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res, 60*(8), 2178-2189. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10786682

Xie, L., Xu, J., Sun, X., Tang, X., Yan, T., Yang, R., & Guo, W. (2019). Apatinib for Advanced Osteosarcoma after Failure of Standard Multimodal Therapy: An Open Label Phase II Clinical Trial. *Oncologist, 24*(7), e542-e550. doi:10.1634/theoncologist.2018-0542

Xu, D., Hu, J., De Bruyne, E., Menu, E., Schots, R., Vanderkerken, K., & Van Valckenborgh, E. (2012). Dll1/Notch activation contributes to bortezomib resistance by upregulating CYP1A1 in multiple myeloma. *Biochem Biophys Res Commun, 428*(4), 518-524. doi:10.1016/j.bbrc.2012.10.071

Xu, J., Zhang, Y., Jia, R., Yue, C., Chang, L., Liu, R., . . . Wang, Q. (2019). Anti-PD-1 Antibody SHR-1210 Combined with Apatinib for Advanced Hepatocellular Carcinoma, Gastric, or Esophagogastric Junction Cancer: An Open-label, Dose Escalation and Expansion Study. *Clin Cancer Res, 25*(2), 515-523. doi:10.1158/1078-0432.CCR-18-2484

Xu, Y., Huang, Z., Lu, H., Yu, X., Li, Y., Li, W., . . . Fan, Y. (2019). Apatinib in patients with extensive-stage small-cell lung cancer after second-line or third-line chemotherapy: a phase II, single-arm, multicentre, prospective study. *Br J Cancer, 121*(8), 640-646. doi:10.1038/s41416-019-0583-6

Yang, X., Liu, D., Murray, T. J., Mitchell, G. C., Hesterman, E. V., Karchner, S. I., . . . Sherr, D. H. (2005). The aryl hydrocarbon receptor constitutively represses c-myc transcription in human mammary tumor cells. *Oncogene, 24*(53), 7869-7881. doi:10.1038/sj.onc.1208938

Yang, Z., Chen, G., Cui, Y., Xiao, G., Su, T., Yu, J., . . . Jin, L. (2019). The safety and efficacy of TACE combined with apatinib on patients with advanced hepatocellular carcinoma: a retrospective study. *Cancer Biol Ther, 20*(3), 321-327. doi:10.1080/15384047.2018.1529099

Ying, H. Y., Gong, C. J., Feng, Y., Jing, D. D., & Lu, L. G. (2017). Serine protease inhibitor Kazal type 1 (SPINK1) downregulates E-cadherin and induces EMT of hepatoma cells to promote hepatocellular carcinoma metastasis via the MEK/ERK signaling pathway. *J Dig Dis, 18*(6), 349-358. doi:10.1111/1751-2980.12486

Younossi, Z., Anstee, Q. M., Marietti, M., Hardy, T., Henry, L., Eslam, M., . . . Bugianesi, E. (2018). Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nat Rev Gastroenterol Hepatol, 15*(1), 11-20. doi:10.1038/nrgastro.2017.109

Younossi, Z. M., Stepanova, M., Rafiq, N., Makhlouf, H., Younoszai, Z., Agrawal, R., & Goodman, Z. (2011). Pathologic criteria for nonalcoholic steatohepatitis: interprotocol

agreement and ability to predict liver-related mortality. *Hepatology, 53*(6), 1874-1882. doi:10.1002/hep.24268

Yu, B. W., Zhang, L. Q., Teng, X. L., Zhang, Y., Zou, L. B., & Ying, H. Y. (2015). Association between the CYP1A1 polymorphisms and hepatocellular carcinoma: a meta-analysis. *Genet Mol Res, 14*(1), 1076-1084. doi:10.4238/2015.February.6.11

Yu, L., Sun, L., Jiang, Y. F., Lu, B. L., Sun, D. R., & Zhu, L. Y. (2012). Interactions between CYP1A1 polymorphisms and cigarette smoking are associated with the risk of hepatocellular carcinoma: evidence from epidemiological studies. *Mol Biol Rep, 39*(6), 6641-6646. doi:10.1007/s11033-012-1469-3

Yu, M. W., Chiu, Y. H., Yang, S. Y., Santella, R. M., Chern, H. D., Liaw, Y. F., & Chen, C. J. (1999). Cytochrome P450 1A1 genetic polymorphisms and risk of hepatocellular carcinoma among chronic hepatitis B carriers. *Br J Cancer, 80*(3-4), 598-603. doi:10.1038/sj.bjc.6690397

Zhang, B., Zhang, Y., Dagher, M. C., & Shacter, E. (2005). Rho GDP dissociation inhibitor protects cancer cells against drug-induced apoptosis. *Cancer Res, 65*(14), 6054-6062. doi:10.1158/0008-5472.CAN-05-0175

Zhao, Z., Wu, H., Wang, L., Liu, Y., Knapp, S., Liu, Q., & Gray, N. S. (2014). Exploration of type II binding mode: A privileged approach for kinase inhibitor focused drug discovery? *ACS Chem Biol, 9*(6), 1230-1241. doi:10.1021/cb500129t

Zheng, R., Qu, C., Zhang, S., Zeng, H., Sun, K., Gu, X., . . . He, J. (2018). Liver cancer incidence and mortality in China: Temporal trends and projections to 2030. *Chin J Cancer Res, 30*(6), 571-579. doi:10.21147/j.issn.1000-9604.2018.06.01

Zhu, A. X., Finn, R. S., Edeline, J., Cattan, S., Ogasawara, S., Palmer, D., . . . investigators, K.-. (2018). Pembrolizumab in patients with advanced hepatocellular carcinoma previously treated with sorafenib (KEYNOTE-224): a non-randomised, open-label phase 2 trial. *Lancet Oncol, 19*(7), 940-952. doi:10.1016/S1470-2045(18)30351-6

Zhu, R. X., Seto, W. K., Lai, C. L., & Yuen, M. F. (2016). Epidemiology of Hepatocellular Carcinoma in the Asia-Pacific Region. *Gut Liver, 10*(3), 332-339. doi:10.5009/gnl15257

Zhuang, B. W., Li, W., Xie, X. H., Hu, H. T., Lu, M. D., & Xie, X. Y. (2019). Sorafenib versus hepatic arterial infusion chemotherapy for advanced hepatocellular carcinoma: a systematic review and meta-analysis. *Jpn J Clin Oncol*. doi:10.1093/jjco/hyz069