UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXXV CICLO DEL DOTTORATO DI RICERCA IN

BIOMEDICINA MOLECULARE

MOLECULAR PROFILING OF LIVER CANCER: GENETIC AND EPIGENETIC VARIATION ANALYSIS FOR DEVELOPING POTENTIAL TARGETS FOR THERAPY

Settore scientifico-disciplinare: BIO/11

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Abbreviations

HCC – hepatocellular carcinoma
HBV – hepatitis B virus
HCV –hepatitis C virus
NAFLD – non-alcoholic fatty liver disease
MAFLD – metabolic associated fatty liver disease
NASH - non-alcoholic steatohepatitis
PNPLA – patatin-like phospholipase domain-containing protein
DNA – deoxyribonucleic acid
RNA – ribonucleic acid
CSC – cancer stem cell
EpCAM – epithelial cell adhesion molecule
AFP – alpha-fetoprotein
CK7 – cytokeratin-7
GS – glutamine synthetase
CTNBB1 – catenin beta-1
TP53 – tumor protein 53
TNM – tumor-node-metastasis
BCLC – Barcelona clinic liver cancer
DNMT – DNA methyltransferase
RASSF1 – ras association domain family member 1
IGF2 – insulin-like growth factor 2
APC – adenomatous polyposis coli
SEPT9 – septin 9
EFNB2 – ephrin B2
GSTP1 – glutathione s-transferase pi
CHRNA3 – cholinergic receptor nicotinic alpha 3
DOK1 – docking protein 1
MGMT – methylguanine methyltransferase
SOCS1 – suppressor of cytokine signaling 1
CIMP – CpG island methylator phenotype
GGT – gamma-glutamyl transferase
EMT – epithelial-mesenchymal transition
AFB1 – aflatoxin B1
EGF – epidermal growth factor
HGF – hepatocyte growth factor
VEGF – vascular endothelial growth factor
RFA – radiofrequency ablation
TACE – transarterial chemoembolization
VEGFR – vascular endothelial growth factor receptor
FGFR – fibroblast growth factor receptor
PDGFR – Platelet-derived growth factor receptor
TKI – tyrosine kinase inhibitor
RTKI – receptor tyrosine kinase inhibitor
NRTKI – non-receptor tyrosine kinase inhibitor
ICI – immune checkpoint inhibitor
PD-1 – programmed cell death protein 1
PD-L1 – programmed-death ligand 1
OS – overall survival
PFS – progression-free survival
AASLD – American Association for the Study of Liver Diseases
EASL – European Association for Study of Liver
APASL – Asia Pacific Association for the Study of Liver
CML – chronic myelogenous leukemia
BRCA – breast cancer
NSCLC – non-small cell lung carcinoma
CRC – colorectal cancer
EGFR – epidermal growth factor receptor
Kras – Kirsten ras proto-oncogene
ES – Edmondson Steiner grading
FBS – fetal bovine serum
SDS – sodium dodecyl sulfate
BCA – bicinchoninic acid
PCR – polymerase chain reaction
MS-PCR – methylation-specific PCR
RT-qPCR – real-time quantitative PCR
gDNA – genomic DNA
bcDNA – bisulfite converted DNA
cDNA – complementary DNA
WB – western blot
SDS-PAGE - SDS polyacrylamide gel electrophoresis
PVDF – polyvinylidene difluoride
FACS – fluorescence-activated cell sorting
PBS – phosphate-buffered saline
5-AZA – 5-Azacytidine
SOR – sorafenib
SAR –saracatinib
DAS – dasatinib
DMSO - dimethyl sulfoxide
MTT - (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
siR-PD-L1 - RNA silencing of PD-L1
PPI – protein-protein interaction
LIHC – liver hepatocellular carcinoma
TCGA – The Cancer Genome Atlas
GEPIA – Gene Expression Profiling Interactive Analysis
SD – standard deviation
SEM – standard error of mean
ANOVA – analysis of variance
TSG – tumor suppressor gene
FIF-HCC – Fondazione Italiana Fegato - HCC
SFK – Src family of tyrosine kinases
LCCL – liver cancer cell lines
TSC1 – tuberous sclerosis complex 1
TSC2 - tuberous sclerosis complex 2
AURKA – Aurora kinase A
HBsAg – hepatitis B surface antigen
MDM2 – mouse double minute 2 homolog
ALK – anaplastic lymphoma kinase
PTEN – phosphatase and tensin homolog
ALL – acute lymphocytic leukemia
MTD – maximum tolerated dose
RTK – receptor tyrosine kinase
NRTK – non-receptor tyrosine kinase

Summary

Liver cancer ranks as the sixth most common malignancy and the third most common cancer-related death worldwide. Hepatocellular carcinoma (HCC) accounts for ~90% of liver cancer cases. The cellular and molecular heterogeneity of HCC tumors can influence disease progression, classification, drug resistance, and clinical outcome of patients. At present, sorafenib (SOR), a dual-target tyrosine kinases inhibitor (TKI), is the main therapy for advanced-stage HCC. However, this drug only offers limited treatment and survival benefits to patients. Therefore, prognostic and predictive biomarkers for advanced-stage HCC and the discovery of new target molecules for therapy are still warranted to improve patients' survival. This study aimed to discover genetic and epigenetic target molecules to discover potential therapeutic modalities that may encompass HCC heterogeneity.

Based on this aim, we worked on three main tasks.

1. To understand the interspatial tumor distribution of DNA methylation in HCC tumors and evaluate epigenetic therapy and HCC heterogeneity

2. To identify molecular targets of therapies that can comprise HCC heterogeneity

3. To evaluate the anti-cancer effect of the inhibition of identified molecular targets

In this project, we used a translational approach, combining data taken from human HCC clinical specimens, *in vitro* model, *in vivo* mouse model (C57BL/6J-TG(ALB1HBV)44BRI/J), and *in silico* analysis. For *in vitro,* we used models of heterogeneity composed of five HCC cell lines under two subtypes: S1/TGFβ-Wnt-activated (HLE, HLF, and JHH6) and S2/progenitor (HepG2 and Huh7), and an immortalized normal hepatocyte (IHH) as control.

To understand epigenetic regulation in HCC, we investigated the suppressor of cytokine signaling 1 (*SOCS1*), one of the reported relevant genes in hepatocarcinogenesis. HCC clinical specimens were evaluated for *SOCS1* DNA methylation and *SOCS1* gene expression. We observed *SOCS1* methylation to be differentially distributed between tissues representing interspatial tumor sections. The effect of 5-Azacytidine (5-AZA), a demethylation agent, was assessed in HCC cell lines. Treatment with a non-toxic concentration of 5-AZA reduced DNMT1 expressions. The extent of reduction was greater in the S1/TGFβ-Wnt-activated subtype cells as compared to S2/progenitor subtype cells. Furthermore, 5-AZA non-toxic

treatment was able to restore *SOCS1* expression in cells at varying extents. The results of this task indicated intratumoral differences in *SOCS1* methylation in HCC and how cellular heterogeneity influences the response to epigenetic therapy.

Working on our preliminary information that cellular heterogeneity may influence cellular response to therapy, we performed a network analysis of available data on HCC heterogeneity. Using an *in-silico* model approach, we were able to identify potential targets for treatments. Network analysis using protein-protein interaction (PPI) analysis identified 16 proto-oncogenes targets. Using the same cellular models mentioned above, we evaluated three treatment modalities, 5-AZA, sorafenib (SOR), and PD-L1 gene silencing. Our results showed that the majority of the 16 targets were highly upregulated in cells belonging to the S2/progenitor subtype. After exposure to treatments, SOR was effective to reduce the expression of the proto-oncogene targets mostly in the S2/progenitor subtype while PD-L1 silencing was able to decrease the expression of targets in all HCC subtypes. This may suggest that the anti-PD-L1 treatment strategy is an effective proto-oncogene regulator, comprising cellular heterogeneity. This could indicate a treatment advantage of PD-L1 modulation on different cellular subtypes. The result of this task adds to the preliminary information of task 1 that liver cancer cellular heterogeneity is relevant to the response to cancer therapies.

In continuation of our work above, we analyzed a direct and indirect interaction of the 16 targets and performed gene enrichment analysis using web tools. Data showed the unique clustering of a group of non-membrane tyrosine kinases. The Src family of kinases (SFKs: *SRC1, FGR, FYN*, and *YES1*) has been reported to have roles in liver cancer formation and metastasis. We analyzed the expression of these SFKs in our (1) *in vitro* models, (2) HCC clinical samples, and (3) tissues from various stages of liver injuries coming from the mouse models. Our results confirmed the dysregulation of these SFKs across various models of carcinogenesis. We then tested two SFK inhibitors: saracatinib (SAR) and dasatinib (DAS), either as a monotherapy or in combination with SOR. Following *in vitro* treatments of 24h, SAR and DAS treatment alone were not significantly toxic to the cells. This was the same observation with SOR treatment alone, indicating a modest reduction in cell viability after treatment. Interestingly, combination therapy between either SFK inhibitors with SOR resulted in a dose-dependent sensitivity to the drugs. Whereas the effect in IHH control cells, the combination of SAR+SOR had a lesser toxic effect compared to DAS+SOR treatment. The result of the wound healing migration assay revealed an enhanced anti-migration effect of the

combination of SAR/DAS +SOR treatment in HCC cell lines as compared to SAR or DAS treatment alone. The result of this task was able to identify the potential of SFK inhibitors in combination with SOR therapy to enhance treatment effects in HCC comprising cellular heterogeneity.

In conclusion, this study highlights HCC response to therapy as cellular subtype dependent. We propose that combination treatments between SFK inhibitors and SOR as a potential treatment modality in improving HCC therapy to encompass cellular heterogeneity.

Chapter I - Introduction

1.1 Hepatocellular carcinoma: a global burden with diverse distribution

Liver cancer is a global health burden and challenge, with an estimated incidence of >1 million cases by 2025 [1]. Between 2020 and 2040, there is an estimated annual increase of 55% in new liver cancer cases. It is also predicted that 1.3 million people might die from liver cancer by 2040, which is 56.4% more deaths compared to estimated deaths in 2020. More than 80% of liver cancer cases are attributed to hepatocellular carcinoma (HCC), cancer in the hepatic parenchyma [2]. It is one of the most common cancers and causes of cancer-related death worldwide [3]. In 2020, HCC contributed to 905,677 new cases and 830,180 deaths worldwide [4]. A similar number between cases and deaths implies high mortality of this malignancy.

Despite the various attempts at prevention and surveillance, diagnosis, and therapy, the global burden of HCC is substantial. Data from 46 countries ranked HCC among the top three causes of cancer death and expanded data considering 90 countries ranked it among the top five [5]. Next to lung cancer, HCC ranked as the second most common cause of premature death from cancer in 2020, with more than 530,000 deaths among persons aged 30 to 69 years [6]. HCC has a strong male predominance, indicated by 2-3 times higher rates in incidence and mortality among men compared to women [4].

In eastern Asia and Africa, the highest risk factor is a chronic infection of hepatitis B virus (HBV), whereas, in western countries and Japan, chronic infection of hepatitis C virus (HCV) is the highest risk factor [7]. Even though the chronic infection of HBV and HCV remain principal factors for HCC development, the prevalence of the metabolic risk factors such as non-alcoholic fatty liver disease (NAFLD) - recently termed the metabolic-associated fatty liver disease (MAFLD) [8] – is increasing, and it may become the major cause of HCC globally [9]. Other risk factors such as aflatoxin-contaminated foods, heavy alcohol intake, excess body weight, type 2 diabetes, and smoking also contribute to the occurrence of HCC [10]. Several of these risk factors contribute to the presence of chronic liver disease. There are over 90% of HCCs develop from cirrhosis brought by any etiology [11,12]. HCC develops with an annual incidence of 1-6% and is a main cause of mortality in cirrhotic patients [13].

Aging is also a strong risk factor for HCC. The highest age-specific incidence reported in individuals is >70 years of age [14]. There is a difference in the age of onset of HCC according

to geographical location and hepatitis virus infection. In areas mainly associated with HCV-induced HCC, like Japan, North America, and European countries, the malignancy tends to occur later in life (median age of onset is >60 years). On the other hand, in areas mainly associated with HBV-induced HCC like Asia and most African countries, HCC is commonly diagnosed in the age range 30–60 years [15].

In a study by Rich *et. al*, on HCC patients in the United States, they evaluated the possible disparity between race and ethnicity in the incidence of HCC. They have observed that there is a higher proportion of Hispanics and Blacks diagnosed at later stages. Compared to non-Hispanic whites, Hispanics had an advanced liver dysfunction with a decreased tumor burden, meanwhile, the non-Hispanic blacks had better liver function but were more often detected symptomatically with a larger tumor stage. They noted that this difference in tumor stage at diagnosis may be partly due to racial/ethnic differences in liver disease etiology and recognition of cirrhosis, HCC surveillance utilization, and access to primary and subspecialty healthcare. The higher incidence of HCC in Hispanics was also noted to be associated with a higher NAFLD prevalence and increased risk of NASH in Hispanics [16] and single-nucleotide variants in *PNPLA3*, linked to NASH-associated HCC [17].

HCC is a malignancy associated with known diverse underlying etiologies that reflect its geographical distribution. Factors such as timing and level of exposure to environmental and infectious risk factors, healthcare resource availability, and the ability to detect earlier stage HCC and availability of potentially curative treatment contribute to the global variations in the incidence and mortality of HCC [18].

1.2 Hepatocellular carcinoma: a malignancy marked by cellular and molecular heterogeneity

HCC carcinogenesis involves a muti-step process that constitutes the transformation of normal hepatocytes into carcinoma. Several factors contribute to its development, namely, genetic predisposition, reciprocal interactions between viral and non-viral risk factors, the cellular microenvironment and various immune cells, and the severity of the underlying chronic liver disease [1].

Tumor heterogeneity pertains to two aspects, (1) intertumoral (tumor by tumor) and (2) intratumoral (within a tumor) heterogeneity. Intertumoral heterogeneity refers to tumors from

different patients where the difference in genotype and phenotype is induced by diverse etiological and environmental factors [19]. Meanwhile, intratumoral heterogeneity is the diversified genomic and biological variations within a patient's tumor lesion influenced by tumor cell evolution under diverse microenvironments linked to different etiologies. In HCC, the observed intratumor heterogeneity has major implications for diagnosis and therapy [20]. In the context of Darwinian clonal evolution selection, a single malignant cell transforms into a functionally heterogeneous HCC tumor with a hierarchically organized tumor cell community, promoting its survival and fitness in response to the various microenvironments [21].

In line with the advances in biotechnologies and medicines, it became more convenient to identify intratumor heterogeneity by investigating DNA, RNA, and protein, simply because the technology available makes it possible to understand the variations. Cellular heterogeneity is fundamental in intratumoral heterogeneity, impacting the progression of tumors. Initially, cellular heterogeneity was observed only by pathologists using microscopes to identify the different cellular subclones that exist in a tumor slice. Eventually, it became possible to determine cellular heterogeneity by immunohistochemical staining [22], but mainly in qualitative parameters. It was noted that the extent of the tumor heterogeneity in HCC tumors is relative to their tumor size. In tumors measuring from 3 to 5 cm, the intratumor heterogeneity according to histologic differentiation grade and proliferative activity can occur up to 64% [23], while for small tumors (<2cm) it can range from 25% to 47% [24]. Eventually, single-cell analytics like single-cell sequencing, single-cell immunoblotting, single-cell mass spectrometry, and single-cell multi-omic techniques provided ways to characterize cellular heterogeneity [25–27].

A particular key population of cancer cells influences intratumoral heterogeneity, known as the cancer stem cells (CSCs). Various cell surface markers have been associated with the identification of HCC CSCs, namely, EpCAM (CD326), CD90 (THY-1), CD24, CD133 (Prominin-1), CD13 (ANPEP), CD44, and CD47 [28,29]. CSCs can self-renew, differentiate, and maintain tumor growth [30], acting as stem cells in a cancerous tissue [31]. They occupy a major portion of the heterogeneous population of cells in a cancer mass. From their stemness and cancerous properties these cells are responsible for the maintenance and propagation of tumor [32]. However, CSCs present an uneven distribution of functionally diverse subpopulations across different regions of a tumor which influences tumor outcome,

aggressiveness, and invasiveness. It was demonstrated, that the interaction of EpCAM+ and CD90+ CSC cells in a tumor can influence sensitivity to imatinib [33]. Reports also on the presence of both EpCAM+ and AFP+ HCC cell populations in a tumor can result in an activated WNT/β-catenin signaling, associated with an increase in tumor growth and invasiveness [34].

A systematized understanding of intratumor heterogeneity was done by Friemel *et. al*. Their study analyzed 23 HCC tumors without pre-medical treatment, looking into 120 tumoral regions. They comprehensively assessed each specimen analyzing tumor morphology, immune phenotype using tumor-associated markers (CK7, CD44, AFP, EpCAM, and GS), and mutational status within the *CTNNB1* and *TP53* genes. The results of their study revealed 87% intratumoral heterogeneity (20 out of 23), indicating only 13% (3 out of 23) of patients showed homogeneity. Data was considered in tumors showing heterogeneity either in morphology alone, or the morphology and immunohistochemical characteristics, or morphology + exposed antigens + mutational status of the *CTNNB1* and *TP53* genes [35]. Despite the small sample size of this study, it provided substantial evidence of the intrinsic heterogeneous characteristics of primary tumors. The existing heterogeneity of primary tumors acts as a driving force of the tumor's clonal evolution, progression, and resistance to chemotherapy [20]. However, chemotherapy can eventually influence the dominance of existing minor or dormant lineages in tumors [36].

Various attempts have been made to categorize HCC heterogeneous tumors and classify them according to shared cellular and molecular profiles. It was Lee and Thorgeirsson who first reported the unrecognized, clinically relevant subclasses of HCC by looking at gene expression profiles of tumors. The underlying molecular traits found in their study led to the stratification of phenotypic subclasses of HCC [37]. The succeeding work of Boyault *et al*., focused on transcriptome–genotype–phenotype profiles of HCC tumors and proposed a classification consisting of six subgroups (G1 to G6) based on their shared clinical and genetic profiles [38]. The works of Hoshida *et al.* looked into clinical parameters such as tumor size, the extent of cellular differentiation, and serum α-fetoprotein levels and were able to suggest a robust subclassification of HCC. Their analysis of the signatures proposed three subclasses: (1) S1, marked by aberrant activation of the WNT signaling pathway; (2) S2, reflected by significant EpCAM positivity and also MYC and AKT activation; (3) S3, tumors classified by hepatocyte differentiation [39]. With these existing subclasses, the work of Caruso *et al*. utilized liver

cancer cell lines to understand the diversity of HCC tumors observed in patients. They concluded that experimental *in vitro* models could be reliable and viable tools to reflect HCC tumors in patients and provide simple approaches to the challenges of HCC biomarker discovery and drug response [40].

The suggested HCC classifications and subtyping are an attempt to create a homogenized clustering of tumors in HCC. This strategy aims to construct rational protocols with targeted therapies and be able to refine prognosis in patients [41]. Despite the continuous advancement in technologies generating coherent pieces of evidence in HCC classifications, there still exists quite a diverse profile of HCC tumors. Thus, making it very challenging to homogenize the clustering. Discoveries and discrete findings prove that the current knowledge on HCC classifications may or may not be able to capture the completeness of HCC heterogeneity. Also, despite the presented benefits of subgroupings and classifications of HCC tumors, intratumoral heterogeneity is still not fully considered in the current actual scoring criteria used in the clinics. Notably, the current staging systems for HCC (e.g. tumor-node-metastasis (TNM) or Barcelona Clinic Liver Cancer (BCLC)) used to classify tumors and determine successful treatment outcome [42], does not consider molecular heterogeneity.

1.3 Hepatocellular carcinoma: its development and molecular players in carcinogenesis

1.3.1 CSC theory and clonal evolution theory

Tumor heterogeneity is explained in two models. First is the “cancer stem cell model”, focused on a subpopulation of tumor cells that are capable of self-renewal and are potentially tumorigenic, known as the CSCs. This model represents a hierarchy of cells where a subset of neoplastic cells feed on the abnormal growth of tissue and can differentiate into specialized tumor cells also as inactive tumor cells [20]. CSCs can influence cellular heterogeneity by imposing a differentiation in the hierarchy, leading to a range of different cell types within a tumor [43]. The other model of tumor heterogeneity is the “clonal evolution model”. This pertains to the assumption that primary tumors arise from a single mutated cell that accumulates progressive mutations during an uncontrolled cell growth [21]. This produces the heterogeneous population of subclones that gain reproductive and survival advantages, which in time promotes dominant variants to gain growth advantages. As compared to the first model,

this model does not present a hierarchy during tumorigenesis, indicating differences in the tumor properties of resulting subpopulations. In the context of HCC treatment, individual CSCs present a rather specific group of cells as therapeutic target [44], while in the clonal evolution model, each abnormal cell must be tackled individually [45]. These two proposed models of heterogeneity are not mutually exclusive. The existence of both in a tumor mass can synergistically promote tumor development and heterogeneity in the hepatocarcinogenesis [20].

1.3.2 Epigenetic players of hepatocarcinogenesis: role of DNA methylation

Rapid advances in molecular medicine have opened new perspectives in dissecting HCC heterogeneity, including epigenetic variations. DNA methylation, one of the most studied epigenetic modifications, controls gene expression by altering the chromosomal structure, DNA conformation, DNA stability, and the function between DNA and protein [46]. It involves the transfer of a covalent methyl group to the C5 position of the cytosine to form 5-methylcytosine by DNA methyltransferases (DNMTs) [47]. There are two main DNA methylation-related modifications in tumors: (1) a genome-wide DNA hypomethylation (decrease in methylation), more related to chromosomal instability, the reactivation of transposable elements or the loss of imprinting, and (2) the hypermethylation (increase in methylation) of the CpG islands in the promoter regions of tumor suppressor genes that leads to their inactivation [48].

Targeting DNMTs to inhibit DNA methylation has been explored as a cancer therapy. The prevention and reversal of methylation in silenced tumor suppressor genes (TSGs) in cancer can lead to the restoration of its function, leading to possible suppression of malignancy. DNMT inhibitors such as 5-Azacytidine and 5-Aza-2′-deoxycytidine are being used to treat hematological malignancies [49] and other cancers [50]. However, in terms of epigenetic treatment, this inhibition of DNMTs can also lead to loss of heterozygosity and global hypomethylation leading to a general decrease in methylation activities that may affect also the normal patterns of gene regulation. Despite this concern, several studies of these DNMT inhibitors have generated outcomes that lead to the reduction of malignancy and improved survival [51]. The integration of this nucleoside must happen during the S phase of the cell cycle during the replication process. Hence, this drug can incorporate itself effectively to actively replicate tumor cells [52].

In HCC, DNA methylation profiling by genome-wide arrays has been explored in both clinical samples and cell lines, showing enormous variations and different clinical associations [53–55]. Various methylated genes have been associated with diagnosis, prognosis, and treatment options, as reviewed in [56]. Villanueva *et al.* analyzed 331 surgically resected HCC and 19 non-tumor tissues, including 9 cirrhosis and 10 normal livers. Their study wanted to understand the epigenetic alterations associated with HCC and understand the role of DNA methylation markers as potential biomarkers for HCC prognosis. Both methylome and transcriptome profiling was done, using Illumina Human Methylation 450 array (Illumina, Inc., San Diego, CA), covering 96% of known cytosine phosphate guanine (CpG) islands and 485,000 CpG, and Affymetrix Human Genome U219 Plate (Affymetrix, Inc., Santa Clara, CA) and miRNA Chip 2.0, respectively. The study was able to confirm, the high prevalence of genes known to be deregulated by aberrant methylation in HCC (e.g., *RASSF1*, *IGF2*, *APC,* and *NOTCH3*) and describes potential candidate epi-drivers (e.g., *SEPT9* and *EFNB2*) [57]. Aberrant methylation of *GSTP1*, *CHRNA3*, and *DOK1* was specifically associated with HCC but not in cirrhotic and normal tissues. Additionally, the hypomethylation of *MGMT* was associated with alcohol intake of HCC patients while hypermethylation of *GSTP1* was associated with HBV infection [58].

Considering hepatocarcinogenesis, *SOCS1* as a tumor suppressor gene was found to be frequently silenced through epigenetic disruption. The incidence of *SOCS1* aberrant DNA methylation was around 60% in HCC tumor specimens [59,60], indicating it is a common event in HCC. The suppressor of cytokine signaling 1 (*SOCS1*), encodes a member of the STAT-induced STAT inhibitor. It is responsible for negative feedback regulation of the JAK-STAT pathway induced by cytokine stimulation [61]. Restoration of *SOCS1* upon methylation leads to a suppressed HCC growth rate and anchorage-independent growth [59].

Evidence on the role of DNA methylation status as a valuable prognostic indicator in HCC is demonstrated by Cheng *et al*. A high CpG island methylator phenotype CIMP (defined as having four or five of 10 commonly methylated genes in HCC) was correlated with worse prognosis, increased TNM stage, increased metastasis and increased gamma-glutamyl transferase (GGT) levels in HCC patients (vs those with less hypermethylated candidate genes) [62]. In addition, epigenetic modifications on DNA methylation can also determine the success of therapy. Chemotherapeutic agents inducing hypomethylation may be beneficial in the short-term, but this strategy may eventually allow progression and recurrence from cancer cells that

survive or are even enhanced by DNA hypomethylation (an independent factor in cancer progression and formation) [63]. Feinberg *et al*. proposed the epigenetic progenitor model of cancer, describing how early epigenetic changes lead to tumor formation and influence heterogeneity and chemoresistance [64].

DNA methylation has prognostic and therapeutic functions in hepatocarcinogenesis. Integrative analyses about their potential as biomarkers for HCC and even heterogeneity should be considered. The easy and cost-effective laboratory tools used to investigate methylation profiles make these markers a convenient target to establish diagnosis, prognosis, and possibly a response to treatment [65]. The existence of drugs that can easily reduce the presence of methylation and subsequently restore expression and activity of aberrant genes can offer potential therapeutic options in managing HCC, probably not as a single agent but in combination with other treatments to minimize toxicity but increase efficiency by targeting multiple aspects of tumor progression [52].

1.3.3 Molecular players and pathways of carcinogenesis

The most frequently mutationally activated oncogene in HCC is β-catenin (CTNNB1), found in 20 to 40% of HCC tumors [66]. This molecule is defined as an HCC driver. In hepatocarcinogenesis, the role of the WNT/β-catenin is associated with lineage specification, differentiation, stem cell renewal, epithelial-mesenchymal transition (EMT), zonation, proliferation, cell adhesion, and liver regeneration [67–69]. It was noted that the presence of β-catenin mutations is associated with chromosome stability [70] and observed more frequently in HCC patients without HBV infection [71]. Additionally, β-catenin mutation-driven HCC develops from non-cirrhotic livers that are not associated with usual HCC risk factors [72]. Studies suggest that tumors that exhibit this type of mutation present a particular set of features such as high differentiation with a homogeneous microtrabeculo-acinar pattern, low-grade cellular atypia, and cholestasis [73].

The incidence of mutation to a particular tumor suppressor gene has been associated with hepatocarcinogenesis. Mutation of *TP53* revealing a G→T transversion at codon 249, resulting in amino acid substitution R249S, in recorded in >50% of the tumors with high exposure to aflatoxin B1 (AFB1) [74,75]. On the other hand, in areas where there is no exposure to AFB1, this type of mutation is found in approximately 20% of HCCs, without specific hotspots of

mutations [70]. This evidence suggests that this type of *TP53* mutation is associated with the estimated level of AFB1 exposure, indicating this carcinogen has a causative role in HCC development. To add, no *TP53* mutations were found in benign hepatocellular tumors [76].

Other mutations such as the *CTNBB1* activation in liver adenomas were shown to be associated with a higher risk of malignant transformation [77]. This suggests that β- catenin activation is a common genetic determinant associated with both benign and malignant tumorigenesis in the liver [41]. A comprehensive review done by Farzaneh and colleagues, identified TGF-β, WNT/ β -catenin, Hedgehog, Notch, EGF, HGF, VEGF, JAK/STAT, Hippo, and HIF as critical pathways involved in hepatocarcinogenesis and targeting these pathways using small molecules in the control of HCC both *in vitro* and *in vivo* models [78].

1.4 Hepatocellular carcinoma: clinical management and treatment

Despite numerous studies for an early diagnosis, the treatment for HCC remains one of the most difficult to cure [79] and is described as a "chemoresistant" tumor [80]. The carcinogenesis complexity increases the burden in the diagnosis while the heterogeneity (tumor extent, patient comorbidities, and severity of liver dysfunction) challenges both the management and treatment [18].

While proven to be potentially curative and improves survival, radical treatments such as surgical resection and liver transplant are considered only for the early-stage HCC [81], which accounts for a small number of HCC cases. Complete surgical removal is not an option for most HCC patients since more than two-thirds of its cases are already in the advanced and metastatic stages at the time of diagnosis [82]. Besides more than 90% of HCC patients have an incidence of post-surgery recurrence [83].

Radiofrequency ablation (RFA) and transarterial chemoembolization (TACE) are options for unresectable HCC cases [84,85]. Both are locoregional techniques that induce necrosis resulting in tumor shrinkage. For TACE treatment, the coupling with targeted delivery of cytotoxic chemotherapy (e.g. doxorubicin, cisplatin, epirubicin) increases tumor response, decreases progression, and improves overall survival [86,87]. However, these available treatments have remained very limited and only a handful can benefit from existing anti-neoplastic therapies. With only 15% of HCC eligible for the potentially curative treatments

[88] majority of HCC patients are in the advanced stage and rely on the modest benefits of targeted treatments.

Sorafenib has the dual-target function to inhibit the tyrosine kinases in angiogenesis and block the serine-threonine kinase *Raf*, which is part of the *Ras/MEK/ERK* signaling pathway in the cancer cell [89]. Sorafenib was reported to extend the overall survival of HCC patients for about 3 months [90]. Meanwhile, lenvatinib, also a multikinase inhibitor targeting *VEGFR1-3*, *FGFR1-4*, *PDGFRα*, *RET*, and *KIT,* showed clinical activity and acceptable toxicity profiles in patients with advanced HCC [91]. Other TKIs inhibitors that were approved only as second-line therapy for HCC are regorafenib [92], cabozantinib [93], and ramucirumab [94].

While the ability of current therapies such as sorafenib and lenvatinib to be multi-targeted gives them an upper hand in providing a significant anti-tumor effect, the same multiple targets are bound for polymorphism-promoting heterogeneity which may lead to chemoresistance and treatment failure. These genetic variants can also be evident in drug delivery systems, signaling pathways, drug metabolism, and cellular processes which are important in understanding resistance and sensitivity to the drug.

In an attempt to further improve treatment options, immunotherapy has been added for advanced stages tumors for potentially better results [95]. Among various approaches of immunotherapies, including adoptive cell transfer, cancer vaccine, and others, the therapy with immune checkpoint inhibitors (ICIs) has shown a remarkable breakthrough in various types of cancers. Targeting mutual interaction (the checkpoint) between immune and other tumoral cells is beneficial since it can be applied in various stages of cancer. In HCC, ICIs targeting the programmed cell death protein 1 (PD-1) such as nivolumab [96] and pembrolizumab [97], and against cytotoxic T lymphocyte antigen 4 (CTLA-4) such as tremelimumab [98,99] had given promising results in HCC patients with or without previous sorafenib treatment.

Up until now, one of the most encouraging clinical trial data for HCC is the combined therapy anti-PD-L1 atezolizumab and anti-VEGFR bevacizumab. The results of the phase 1b trial GO301240 (NCT02715531) for unresectable HCC resulted in progression-free survival (PFS) of 5.6 months of combined therapy compared to 3.4 months in atezolizumab monotherapy alone [100]. This study was then followed by the IMbrave150, a global, open-label, phase 3 trial, involving 501 unresectable HCC patients at 111 sites in 17 countries. This study looked into the antitumor effects of this combined therapy compared to sorafenib alone, resulting in a

median PFS of 6.8 vs 4.3 months for combination therapy vs sorafenib, as well as a better overall survival (OS) of 19.2 vs 13.2 months. This study concluded that in patients with unresectable HCC, atezolizumab combined with bevacizumab resulted in better OS and PFS outcomes than sorafenib [101,102]. This prompted their use as first-line treatments in advanced HCC [103]**.** Different combination therapies involving ICIs work on (1) the combination of ICIs (either in the tumor cell or in the immune cell) and anti-angiogenesis drugs targeting VEGFR in the vascular endothelial cell, or (2) a combination of ICIs and multi-kinase inhibitors (MKIs) found in endothelial cells and tumor cells and (3) combination of two ICIs targeting immune cells [104].

To date, the guideline*s* for HCC management are acknowledged by several important hepatology societies, such as the American Association for the Study of Liver Diseases (AASLD), the Asian Pacific Association for the Study of the Liver (APASL), and the European Association for the Study of the Liver (EASL) [105–107]. So far, the guidelines from the EASL-BCLC for HCC classification, prognosis, and treatment protocol are the most followed guideline among physicians. However, clinicians can be independent to indicate single or combination therapies for patients to produce better outcomes [108,109].

1.5 Hepatocellular carcinoma: challenges and current gaps to fill

Even though molecular classifications of HCC have been widely studied, unlike other cancers, the utility of individual-specific molecular biomarkers for prognosis and response to treatment is non-existent in HCC. To date, AFP remains the most effective serum biomarker for HCC diagnosis [105]. In cancers like chronic myelogenous leukemia (CML), breast cancer (BRCA), non-small cell lung carcinoma (NSCLC), and colorectal cancer (CRC), biomarkers for diagnosis and treatment are already utilized in clinics. A majority of CML patients with BCR-ABL oncoprotein fusion dictate the favorable response to tyrosine kinase inhibitors (TKIs), like imatinib [110]. For breast cancer, the overexpression of Her2 increases the clinical benefit of a monoclonal antibody, trastuzumab [111]. In the case of non-small cell lung carcinoma (NSCLC), clinical responsiveness to a TKI, gefitinib is determined by specific mutations in the epidermal growth factor receptor (*EGFR*), making mutational screening for this proto-oncogene as a standard of care [112]. On the other hand, the absence of mutation in wildtype *Kras* increases sensitivity to cetuximab in metastatic colorectal cancer patients [113].

Despite the obvious benefits of molecular testing to determine gene/protein profiles for targeted therapy for HCC, there is still the possibility of persistent disease progression in patients after initial sensitivity to treatment. This can eventually lead to poor clinical outcomes in patients [114]. Multiple factors such as microenvironment and intratumor heterogeneity most likely explain this result. The existence of other subclones in a single tumor influences the eventual resistance of the tumor to the matching targeted therapy. Giving evidence that targeting one specific abnormality will not be sufficient to eradicate the totality of tumor cells [115,116].

Early diagnosis is still an effective strategy to manage HCC. However, since most HCC cases are diagnosed in advanced stages, successful therapy is being challenged by heterogeneity and cell resistance, in addition to a rather poor liver function of the patients. It is necessary to consider multi-target approaches to encompass the diverse nature of the tumor and the involvement of multiple cellular processes. Computational approaches in combination with -omics (e.g., transcriptomics, metabolomics) and various disease models cited above may give us more tools to manage drug resistance, especially in sorafenib treatment.

To date, it is rather clear that the combination therapies, like anti-PD-L1 and anti-VEGF, can be the most potent strategy for HCC treatment. It is important to note that the use of combination therapy to specifically target cancer-promoting cells and reduce toxicity has become a cornerstone for cancer treatment [117]. Also, in cases where monotherapy is ineffective, combination therapy can be approached in an additive and synergistic manner to control cancer growth [118].

Chapter II - Aims of the study

Hepatocellular carcinoma is a cancer marked by vast molecular and cellular heterogeneity. Several transcriptomic studies have identified unique molecular signatures that have clustered HCC tumors into different subtypes. Inferring from HCC molecular signatures and cellular heterogeneity, this study aims to understand both similarities and differences in HCC tumors to discover genetic and epigenetic markers for therapy.

Three major tasks were identified to attain the objectives of this study.

TASK 1: Evaluation of the distribution of *SOCS1* DNA methylation in HCC tumors and the potential of epigenetic *SOCS1* modulation in *in vitro* models of heterogeneity.

TASK 2: Identification of potential molecular targets of therapy through the utilization of bioinformatic analysis and evaluation in *in vitro* models of heterogeneity.

TASK 3: Characterization of unique targets of therapy and evaluation of their anti-cancer effects as monotherapy or combination treatment.

Chapter III - Materials and Methods

3.1 Human clinical tissue samples

A total of 95 HCC patients from both Vietnam (n=52) and Italy (n=43) undergoing partial hepatectomy were included in this study. From each patient, different portions of the liver composed of non-tumoral, peritumoral/peri-HCC, and tumoral/HCC tissues were collected. A standardized sample collection procedure (size, type of tissues, and storage condition) between both specimen collection sites was executed. Fresh liver tissues were immediately collected and processed for storage either by suspension in RNAlater solution (Invitrogen, Carlsbad, CA, USA) or snap-frozen in liquid nitrogen and then stored at −80◦C. Simultaneously, corresponding portions of the liver tissues were fixed in formalin and embedded in paraffin. Fixed tissue slices were stained with hematoxylin and eosin (HE) and immunostaining and histological analysis was done by two independent pathologists from University Medical Center (UMC), Ho Chi Minh, Vietnam, and Azienda Sanitaria Universitaria Giuliano Isontina (ASUGI), Trieste, Italy.

For inclusion criteria, the study only enrolled adult patients with a confirmed HCC diagnosis and no other hepatic malignancy or other malignant disease. It was necessary also that these patients have available non-tumoral, peri-tumoral, and tumoral tissues collected for surgical resection to be included in the study. While for exclusion criteria, pediatric HCC patients, patients with other hepatic malignancies or other malignant diseases, and patients with unavailable non-tumoral, peri-tumoral, and tumoral tissues were excluded from the study.

Patients from the Vietnamese cohort were composed of 43 male and 9 female HCC patients. The mean age of the patients in this study population is 58 years old. While in the Italian cohort, there were 33 males and 10 females, with a mean age of 66 years old. In terms of the etiology of the malignancy, the Vietnamese cohort has 33 HBV (63%), 9 HCV (17%), and 10 metabolic/alcohol associated (19%) patients. On the other hand, the Italian cohort has 7 HBV (16%), 14 HCV (33%), and 18 metabolic/alcohol associated (42%) patients. Complete clinic-pathological parameters of the study cohorts are listed in the report of Luong *et. al* [119].

The diagnosis of patients was established on international criteria together with its Edmondson Steiner (ES) HCC grading, tumor parameters, laboratory results, and other clinical findings. The patient's participation in the study was accompanied by duly signed informed consent. The

study's protocols were approved by the ethical committees of the UMC Ho Chi Minh City no. 240/DHYD-HDDD (for Vietnam) and the Comitato Etico Regionale Unico of the Friuli Venezia Giulia, Prot. No. 18854 (for Italy).

3.2 Transgenic mouse liver tissue samples

An animal model for liver damage and carcinogenesis was used in this study. Liver tissues from HBV-transgenic mice C57BL/6J-TG(ALB1HBV)44BRI/J (HBV-TG) [120] together with its wild-type counterpart (C57BL76J) (WT) were collected. Different stages of liver damage were observed indicating inflammation, early hepatic injury, pre-neoplastic lesion, and neoplasia at 3-, 6-, 9-, and 12-months old mice, respectively. Mouse models were maintained at the animal facility of the University of Trieste. Research experiments on the use of mouse models were done following the Guide for the Care and Use of Laboratory Animals. Corresponding protocols and animal studies were reviewed and approved by the ethics committee of the University of Trieste and by the responsible administration of the Ministry of Health of the Republic of Italy (D 699/2020-PR). At least 3 liver sections were harvested from the right and left liver lobes. Tissues were cut and immediately stored on ice and then kept in a -80^{0}C freezer. In the presence of visible nodules in the liver of TG mouse models, nodular and non-nodular sections were cut and collected.

3.3 Cell lines and cell culture maintenance procedures

We selected representative cell lines that correspond to the different subtypes of HCC tumors. Six cell lines, which consisted of 1 immortalized hepatocyte (IHH) and 5 HCC cell lines, were used for *in vitro* analysis. The HCC cell lines HLE, HLF, and JHH6 were classified under the subtype 1/transforming growth factor beta–Wingless related integration site (S1/TGFβ-Wnt) activated subtype and HepG2 and Huh7 were classified as subtype 2 (S2/progenitor subtype) [40]. All cell lines were grown in their respective culture media supplemented with 10% (v/v) fetal bovine serum (FBS), 1% L-glutamine, and 1% antibiotics. Dulbecco's Modified Eagle's Medium (DMEM)-F12 medium was used for the immortalized hepatocytes IHH with additional supplements of 1 μM dexamethasone, and 5 μg/mL insulin. DMEM medium (high glucose) was used for HCC cells, except for JHH6 which was cultured in Williams' E medium.

Cells were maintained at 37 ◦C in a humidified 5% CO2 incubator. Routine cell expansion was performed using 0.05% trypsin detachment when cells achieved 80% cell confluency.

Human HCC cell lines Huh7 (JCRB0403) and JHH6 (JCRB1030) were obtained from the Japan Health Science Research Resources Bank (HSRRB, Tokyo, Japan). The HepG2 cell line was obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER, Brescia, Italy). While cell lines HLE and HLF were kindly provided by the laboratory of Dr. Gianelli of the National Institute of Gastroenterology "S. De Bellis" Research Hospital, Bari, Italy. IHH was kindly provided by Dr. Trono (Lausanne, Switzerland) [121]. Both HepG2 and Huh7 are hepatoblast-like and well-differentiated HCCs. HepG2 cells were derived from a tumor of a 15-year-old male, Caucasian patient, while Huh7 was from a 57-year-old male, Asian patient. HLE, HLF, and JHH6 cell lines are mesenchymal-like, undifferentiated HCCs. Both HLE and HLF were derived from a 68-year-old, male, Asian patient. Among all HCC cells, the JHH6 cell line was described as non-tumorigenic and is derived from a 58 years old female, Asian patient.

3.4 Isolation of genomic DNA from human tissue

Genomic DNA (gDNA) extraction was performed using the EZ DNA Methylation-Direct™ Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions. Briefly, in a microcentrifuge tube, 0.5 mg of tissues were lysed in a solution of digestion buffer, proteinase K, and water for 20 min at 50◦C. After incubation, the contents of the reaction were mixed thoroughly and then centrifuged for 5 minutes at 10,000 x g. The supernatant containing the DNA was collected for subsequent DNA analysis procedures.

3.5 Total RNA extraction from solid tissue samples and cell lines

Total RNA was extracted from all sample types using Tri Reagent® (Sigma–Aldrich, St Louis, MO, USA) according to the manufacturer's protocol. Tissue homogenization was done by potter elvehjem tissue grinders and cell scraping for solid tissue and cell lines, respectively. All homogenates were suspended in 500-1000 μL of Tri Reagent®, followed by phase separation, RNA precipitation, washing, drying, and RNA pellet dissolution. RNA was quantified at wavelength 260 nm in a spectrophotometer (Beckman Coulter, Brea, CA, USA) and RNA purity was evaluated according to the Minimum Information for Publication of Quantitative

Real-Time PCR Experiments (MIQE) guidelines by measuring the ratio A260/A280 with an appropriate purity value between 1.8 and 2.0 [122]. The integrity of RNA was assessed on standard 1% agarose/formaldehyde gel.

3.6 Protein extraction from cell lines

Total protein was extracted either by direct cell lysis or by protein extraction procedures in samples stored in TRI Reagent®. For the lysed cells, samples were suspended in cell lysis buffer and were further homogenized, centrifuged, and the supernatant containing the protein was collected. For samples suspended in TRI Reagent®, after collection of the organic phase, isopropanol was added for protein precipitation, followed by washing and drying. The retrieved protein pellet was resuspended in 1.0% SDS (sodium dodecyl sulfate). Protein concentration was determined by the bicinchoninic acid protein assay (BCA).

3.7 Bisulfite conversion of genomic DNA

Extracted gDNA was subjected to bisulfite conversion using the EZ DNA Methylation-Direct™ Kit (Zymo Research, Irvine, CA, USA). Approximately 500 ng of DNA from the lysed supernatant was used for bisulfite conversion. Briefly, 200–500 ng gDNA was incubated in the conversion reagent and then treated with a binding buffer in a spin column. Bisulfite-converted DNA (bcDNA) was then subjected to desulphonation and cleaned up using a wash buffer. Bisulfite-converted DNA (~10 µL) was eluted and collected for methylation-specific PCR (MS-PCR).

3.8 Methylation-specific PCR (MS-PCR)

MethPrimer 2.0 Primer Design© web tool [123] was used to determine MS-PCR primers for this study, covering the region around nucleotide 500–700 of CpG island 2 of the *SOCS1* transcript (NM_003745.2) (Figure 1A). This CpG island included exon 2 of the SOCS1 gene. The primers covered at least 23 CpG sites and were about 200 bp in size. Primer sequences are listed in Table 1.

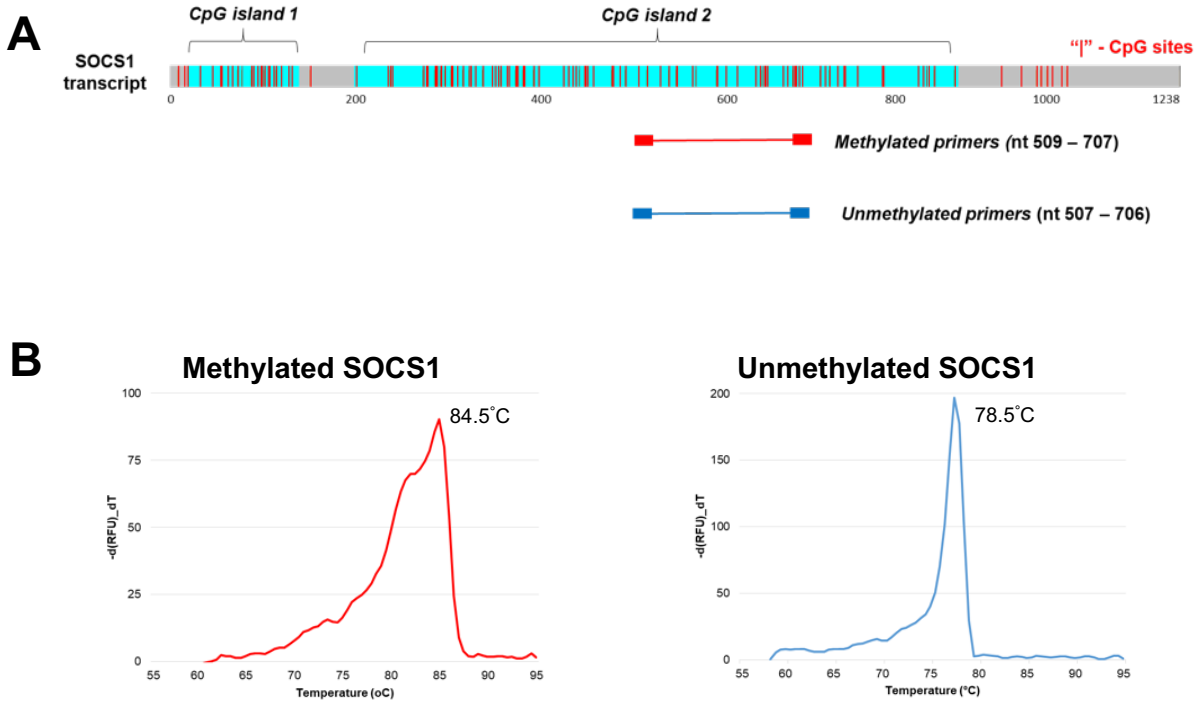


Figure 1. Methylation-specific PCR for *SOCS1* gene. (**A**) The target region of *SOCS1* methylation analysis is located in CpG island 2 of *SOCS1* transcript (NM_003745.2). MS-PCR primers pairs were designed by MethPrimer 2.0 Primer Design© web tool. (**B**) The melting curve graph of MS-PCR detects specific melting peaks of methylated-specific and unmethylated-specific *SOCS1* primers (M = 84.5 °C; UM = 78.5 °C).

Methylation-specific PCR (MS-PCR) was carried out in a 15 µL reaction volume containing 100 ng bcDNA, 1X Power-Up SYBR Master Mix (Thermo Scientific, Waltham, MA, USA), and 250 nM of methylation-specific forward and reverse primers. The presence of a methylated-SOCS1 sample was indicated by the PCR amplification using the methylated-SOCS1 primer with a melting peak temperature of 84.5 ◦C. Accordingly, an unmethylated sample was shown by the detection of the PCR amplification using an unmethylated-SOCS1 primer with a melting peak temperature of 78.5 ◦C (Figure 1B). Partially methylated samples were defined by positive detection of PCR amplification in both primers and the appropriate corresponding melt peaks. Purified gDNA from a human methylated and non-methylated control set (Zymo Research) was used as controls for the methylation analysis.

3.9 Reverse transcription-quantitative real-time PCR (RT-qPCR)

Reverse Transcription (RT) was performed to obtain cDNA from 1 μg of purified RNA with the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's protocol. Real-time PCR was performed according to the SYBR Green Supermix protocol (Bio-Rad Laboratories, Hercules, CA, USA). PCR amplification was carried out in a 15 μL reaction volume containing 25 ng cDNA, 1 × iQ5 SYBR Green Supermix, and 100–250 nM of gene-specific forward and reverse primers. The reaction was run in CFX 9600 real-time PCR system (Bio-Rad). The primer sequences are designed using Beacon Designer 7.9 Software (PREMIER Biosoft International, Palo Alto, CA, USA) for the detection of the desired gene and are listed in Table 1.

Table 1. List of primer pairs used in this study.

Gene Target	**Sequence F (5′ → 3′)**	**Sequence R (5′ → 3′)**	**Ref.**
MS-PCR			
SOCS1-methylated	ATGGTTTCGGGATTTACGAGT	TAACCACGATACGCTAACGAC	ts
SOCS1-unmethylated	AGATGGTTTTGGGATTTATGAGT	AACCACAATACACTAACAACA	ts
Gene Expression			
ACTB	CGCCGCCAGCTCACCATG	CACGATGGAGGGGAAGACGG	ts
SOCS1	CCCTTCCAGATTTGACCG	ATGGTTCCAGGCAAGTAA	ts
DNMT1	CCATCAGGCATTCTACCA	CGTTCTCCTTGTCTTCTCT	[124]
PD-L1	AAAGTCAATGCCCCATACAA	ACATGTCAGTTCATGTTCAGAG	[125]
Molecular Targets			
YAP1	CAATAGCTCAGATCCTTTCCT	TAGTATCACCTGTATCCATCTC	[126]
AURKA	GAGAATTGTGCTACTTATACTG	GGTACTAGGAAGGTTATTGC	ts
FGR	GGCCCGGCCTGCAT	TTGATGGCCTGAGAGGAGAAG	[127]
EGFR	AGGCACGAGTAACAAGCTCAC	ATGAGGGACATAACCAGCCACC	[128]
MET, HGFR	GGGCACCGAAAGATAAACCTCT	GACATTCTGGATGGGTGTTTCC	[129]
YES1	ACAGCAAGACAAGGTGCAAA	GTAAACCGACCATACAGTGCAG	[130]
PLZF, ZBTB16	TCACATACAGGCGACCACC	CTTGAGGCTGAACTTCTTGC	[131]
DCUN1D1	CTGGAGGACACCAACATG	TTCACTAGATTGTGTGAAGATC	[132]
ASV, SRC1	CGCTGGCCGGTGGAGTG	CCAGCTTGCGGATCTTGTAGT	[133]
PRKCA	GTGGCAAAGGAGCAGAGAAC	TGTAAGATGGGGTGCACAAA	[134]
MDM2	TTATTAAAGTCTGTTGGTGCA	TGAAGGTTTCTCTTCCTGAAG	[135]

FOS	CCGGGGATAGCCTCTCTTAC	GTGGGAATGAAGTTGGCACT	[136]
CBL	TGCCAAAACTGCCACCTGGGG	GGGCTGCGGCCAAATTCCCT	[137]
FYN	GGACATGGCAGCACAGGTG	TTTGCTGATCGCAGATCTCTATG	[138]
JUN	AAGTAAGAGTGCGGGAGGCA3	GGGCATCGTCATAGAAGGTCG	[139]
EPS15	CCTGTTGCAGATTTCTCTG	TCATCTTGAAGATCCTGAAC	[140]
Mouse model Targets			
Src/pp60c-src	GTTGCTTCGGAGAGGTGTGGAT	CACCAGTTTCTCGTGCCTCAGT	[141]
Zbtb16/PLZF	CCCAGTTCTCAAAGGAGGATG	TTCCCACACAGCAGACAGAAG	[142]

ts: this study; MS-PCR: methylation-specific PCR.

3.10 Western blot analysis

Protein expressions from treated cells were evaluated using Western blot (WB) analysis. A total of 10 µg of protein lysates was loaded onto 10% polyacrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then wet-transferred onto a polyvinylidene difluoride (PVDF) membrane. Following blocking, membranes were washed and incubated with primary antibodies against the following antibodies: DNMT1 (Abcam, ab19905), SOCS1 (Santa Cruz, E-9, sc-518028), and c-Src (sc-8056, Santa Cruz Biotech) for 24 h. Anti-actin (A2066, Sigma-Aldrich) was used as a housekeeping protein. Secondary antibodies were anti-mouse IgG HRP (Dako-p0260) and anti-rabbit IgG HRP (Dako-p0448), depending on the first antibody. Membranes were washed and then exposed to ECL Plus WB detection system solutions (ECL Plus Western Blotting Detection Reagents, GE-Healthcare Bio-Sciences) to obtain a peroxidase reaction. The blots were visualized using a C-Digit blot scanner and analyzed using Image Studio™ Vers. 5.2 Acquisition software (LI-COR Biosciences). Protein relative quantification was performed after the densitometric analysis of bands *vs* actin in each sample.

3.11 Flow cytometry

The presence of CSC surface marker antigens was detected using antibodies CD90/THY- 1 (Clone 5E10, Stem Cell Technologies, VA, Canada), CD133/PROM1 (clone AC133, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and CD24 (clone 32D12, Miltenyi Biotec). A proposed epithelial CSC marker, CD326/EpCAM (Clone (VU-1D9, Santa Cruz, Dallas, TX, USA) was also used. Additionally, CD13/ANPEP (Clone WM15, Abcam, Cambridge, United

Kingdom) was also included in this study as a surface marker for dormant CSCs. After detachment, at least two million cells per mL were incubated with specific first antibodies for 60 min on ice in the dark. After two washes with PBS containing 0.5% bovine serum albumin (BSA) and 3 mM EDTA, when necessary, the cells were then incubated with fluorescence-conjugated secondary antibody for 60 min on ice in the dark. Flow cytometric analysis was performed immediately in a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA). Ten thousand events were analyzed per sample. An IgG anti-mouse antibody was used as the control.

3.12 Cytotoxicity evaluation of chemical inhibitors

For *in vitro* treatment, four inhibitors were evaluated, consisting of the following: 5-Azacytidine (5-AZA), an epigenetic therapy acting as a DNA methyltransferase (DNMT) inhibitor; sorafenib (SOR), a tyrosine kinase inhibitor; and two Src family of tyrosine kinase (SFK) inhibitors saracatinib (SAR) and dasatinib (DAS).

Each cell line was seeded at 25,000 cells/cm^2, except for JHH6 at 12,500 cells/cm^2. Cytotoxicity experiments were performed to define the lethal concentration (LC_{50}) of 5-AZA, SOR, SAR, and DAS. For the evaluation of 5-AZA (A2385, Sigma-Aldrich, St. Louis, MO, USA), each cell line was treated with concentrations ranging from 2 µM to 5 mM, while for the evaluation of SOR (Nexavar®, Bayer, Leverkusen, Germany), the cell lines were exposed to concentrations from 1 to 80 µM.

For the evaluation of SFK inhibitors, all cells were treated with a concentration of 0.02 to 5.0 µM of SAR or DAS. Additionally, combination treatment was evaluated for SFK inhibitors and SOR. In these experiments, the cells were treated with the same range of concentration of SAR and DAS with an addition of 10 µM of SOR in the treatments. The DMSO concentration was calculated to be 0.1% in the treatment assays. Cell viability was evaluated after 24 h of drug exposure using the 3(4,5-dimethyl thiazolyl-2)-2,5 diphenyltetrazolium assay (MTT, Sigma Aldrich) to determine the LC_{50} of the drug to each cell line.

3.13 PD-L1 mRNA silencing by small interference RNA (siRNA)

Gene-silencing experiments for PD-L1 were performed using 20 nM of siRNA PD-L1 (Hs siRNA against CD274 (Thermo Fisher, Waltham, MA, USA). The siRNAs were transfected into cells using siLentFect^{TM} Lipid Reagent (170–3362, Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Control siRNA (sc-37007, Santa Cruz Biotech, Dallas, TX, USA) was included in each assay. Cells were exposed to siRNA for 48 h, followed by cell collection for RNA extraction.

3.14 Wound scratch assay

Cells were plated in a 12-well plate with an initial concentration of 37,500 cells/cm^2. After 24 hours from cell plating, a longitudinal scratch (wound area) was introduced to the monolayer of cells in each well, by using the end of a white pipette tip. Untreated (CTRL) and treatment set-ups: DMSO, SOR, SAR, SAR+SOR, DAS, and DAS+SOR were prepared in duplicates. In each replicate, we identified at least 3 marked spots along the wound area. These marked spots were viewed and photographed using an optical microscope (Leica, Solms, Germany). Photos were taken from 0, 24, 48, and 72 hours after treatment to record the area of the wound closure.

Photos were first processed using a program, Fiji (Fiji is just ImageJ) (University of Winconsin-Madison, USA) [143] to generate a 1-dimensional image. The program carefully defines and delineates the area where there are no visible cells in the transformed image. This process allows us to define the wound area and assess wound closure by comparing the difference between 0 to 72 hours of treatment. This data is computed to an area of the percentage (%) of wound closure, using this formula: $((t_0-t_h)/t_0))$ x 100%, where t_0 is the area of the wound measured immediately after scratching (0 hour) and t_h is the area of the wound measured "h" hours after the scratch is performed (24, 48 and 72 hours) [144]. Results were presented as a mean (± SD) of measurements of the area of the percentage of wound closure in the 6 marked spots recorded.

3.15 Network analysis for the identification of molecular targets

We investigated published datasets of HCC transcriptomic profiles as presented by Boyault *et al*., and Hoshida *et al*. [38,39]. The two publications proposed groups and subtypes for HCC

based on the similarity of cellular and molecular signatures of tumors. Using these datasets, a protein-protein interaction (PPI) analysis was done using Cytoscape [145] to select common proteins from the PPI network. The gradual screening to select candidate targets was done by excluding housekeeping genes and focusing on genes that were involved in cancer promotion (proto-oncogenes). The clinical association and significance of each proto-oncogene to LIHC (liver hepatocellular carcinoma) were plotted into data from The Cancer Genome Atlas (TCGA) and the Genotype Tissue Expression (GTEx) portals [146,147], and visualized by the Gene Expression Profiling Interactive Analysis (GEPIA) online tool [148]. Figure 2 shows a diagram of the *in-silico* strategy used in this study, while the generated PPI networks from datasets are shown in Appendix.

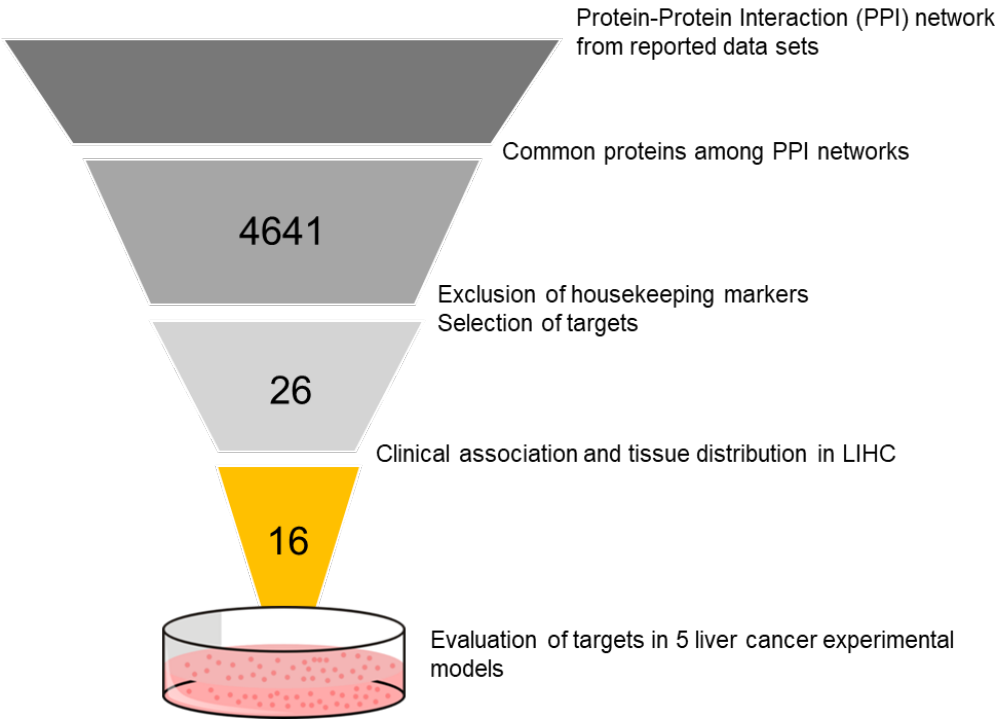


Figure 2. *In-silico* and validation approach to discover potential targets for HCC therapy.

3.16 Statistical analysis

Statistical tests were carried out using the software GraphPad Prism version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). All mRNA data were presented as median (95% CI). For the comparison of mRNA expressions between non-tumoral, peri HCC and HCC tissues, Kruskal Wallis non-parametric test was performed, which was followed by Dunn's multiple comparison test to compare the difference between groups (non-tumoral vs peri-HCC; peri-

HCC vs HCC; non-tumoral vs HCC). For the comparison of the mRNA expressions in the liver tissues of the mouse models, one-way analysis of variance (ANOVA) was used, followed by Newmans-Keul multiple comparison test to compare between groups. For the correlation of methylation and clinicopathological parameters the following tests were performed, Student's t-test, contingency chi-square, and Fisher's exact test, following normality test and are further described in Table 2. Results of the *in vitro* experiments (mRNA and protein expression data) were obtained from at least three independent experiments and are expressed as mean ± SD. Corresponding statistical tests and their significance were set to *p*-value indicated as * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

Chapter IV - Results

4.1 TASK 1: Evaluation of the distribution of *SOCS1* DNA methylation in HCC tumors and the potential of epigenetic *SOCS1* modulation in *in vitro* models of heterogeneity.

The initiation and progression of human cancers is a result of alterations that can be in the form of epigenetic aberrations. For instance, DNA methylation in promoter regions of tumor suppressor genes (TSGs) allows transcriptional silencing that leads to deregulation of many cellular processes that contribute to cancer [149]. Several studies have suggested that methylation of multiple tumor suppressor genes in HCC may contribute to its pathogenesis [150–152]. In TASK 1, we aimed to understand the interspatial distribution of particular epigenetic dysregulation in HCC tumors. We investigated DNA methylation of *SOCS1*, reported to have transcriptional inactivation in HCC progression. Also, we explored the potential of a reprogramming dose of a DNMT inhibitor to revert the expression of *SOCS1* and understand these epigenetic regulations to the heterogeneity of HCC.

4.1.1 SOCS1 methylation in HCC specimen

Total gDNA was extracted from different portions of the liver tissues composed of non-tumoral, peri-HCC, and HCC tissues. In the Italian cohort, the proportion of *SOCS1* methylated samples was observed to be more in HCC tissues as compared to peri-HCC and non-tumoral tissues ($p < 0.001$). In HCC tissues, 54% of the tissues analyzed have methylated *SOCS1*. In contrast, non-tumoral and peri-HCC livers only had 14% and 13% methylated *SOCS1*, respectively. There was a higher frequency of unmethylated *SOCS1* in non-tumoral and peri-HCC tissues as compared to HCC tissues (non-tumoral: 62%; peri-HCC: 53% and HCC: 25%). Tissues with partially methylated *SOCS1* were noticed only in some samples (non-tumor: 24%; peri-HCC: 34% and HCC: 21%) (Figure 3A).

Interestingly, when we evaluated the *SOCS1* methylation in the Vietnamese cohort (data was collected in collaboration with Dr, Hoang Anh Vu and Dr. Luong Bac An, University of Medicine and Pharmacy at Ho Chi Minh City, Vietnam). Our preliminary assessment of results showed a seemingly higher incidence of methylated *SOCS1* in non-tumoral tissues, accounting for 50% of the samples analyzed. The frequency of methylated *SOCS1* was less in peri-HCC

and HCC tissues (21% and 31% respectively). Interestingly, there were a higher percentage of unmethylated *SOCS1* in peri-HCC and HCC tissues as compared to non-tumoral tissues (42% and 44% vs 13% respectively). Partially methylated tissues were also observed in non-tumor, peri-HCC, and HCC samples (38%, 37, and 26%, respectively) (Figure 3B). However, we recommend that this preliminary report be validated using the Italian cohorts as control and/or DNA methylation-sequencing to identify the correctness of the methylation profile. Once validated, the data from the Vietnamese cohort can be further assessed with the clinicopathological association to better understand factors influencing the incidence of *SOCS1* methylation in HCC tumors.

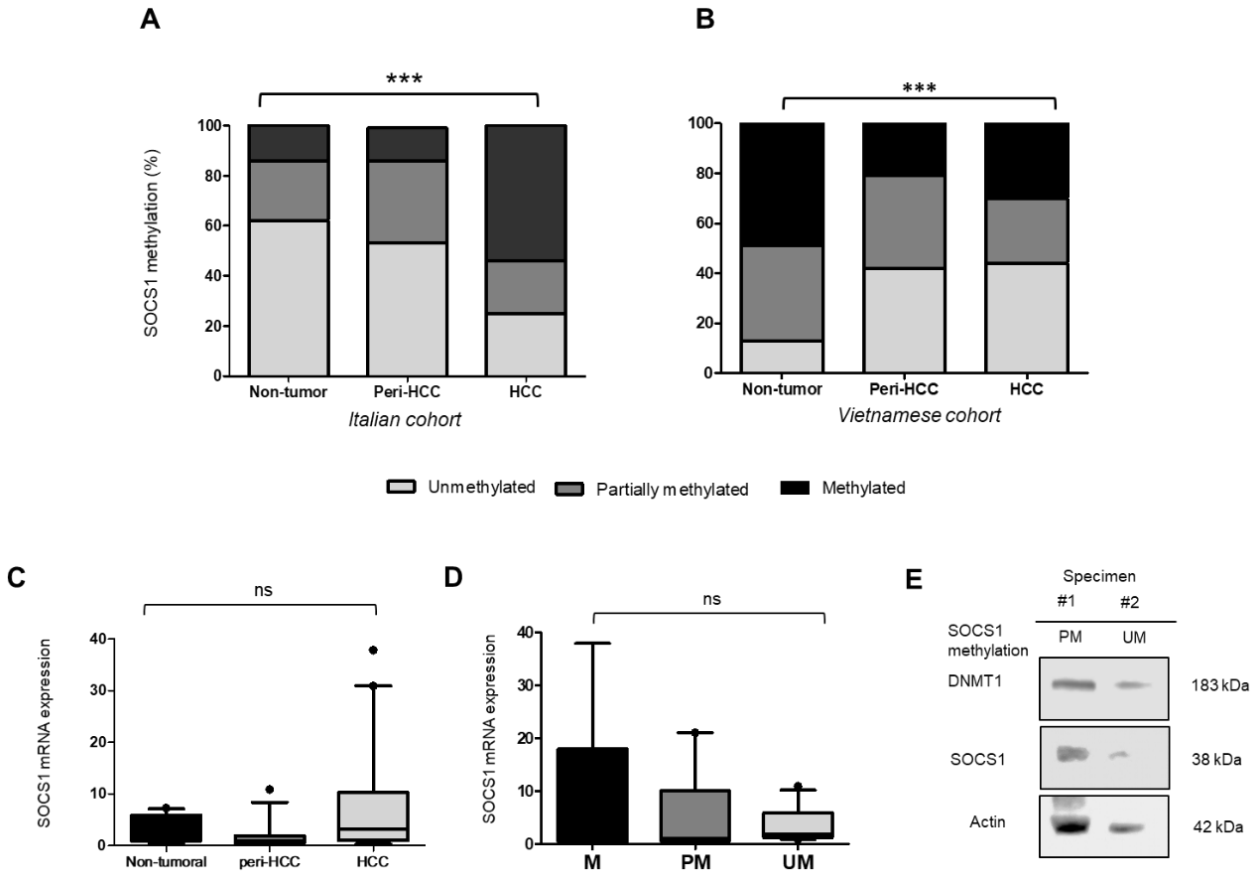


Figure 3. *SOCS1* methylation and mRNA expression in HCC clinical samples. Distribution of *SOCS1* methylation status among HCC liver tissues from (**A**) Italian Cohort (60 tissues) and (**B**) Vietnamese cohort (125 tissues). Data was presented in % value. Statistical analysis for data in Figure 3A and 3B: *** $p \leq 0.001$, using Chi-square test to compare the groups in graph A and B. (**C**) Distribution of *SOCS1* mRNA expression among different tissue samples analyzed by quantitative RT-PCR. (**D**) No correlation between *SOCS1* methylation status and mRNA expression (PCR-based analysis, left). Data from C and D are based on the results of samples from the Italian cohort. Statistical analysis for data in Figure 3C and 3D: ns, using non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test to compare data of non-tumoral, peri-HCC and HCC tissues (**E**) Representative blots of PM and UM clinical specimens showing the protein expressions of DNMT1 (183 kDa) and SOCS1 (38 kDa). ns: not significant. MS-PCR: methylation-specific polymerase chain reaction; M: methylated *SOCS1*; PM: partially methylated *SOCS1*; UM: unmethylated *SOCS1*

We checked the association of *SOCS1* methylation status and clinicopathological data only in the Italian cohort. *SOCS1* methylation status was positively associated with aging ($p < 0.05$). There is a higher incidence of methylated *SOCS1* in moderately differentiated tissues, Edmonson-Steiner histological grade 2 and grade 3 ($p < 0.05$), but it was not correlated with etiology, tumor size, Child–Turcotte–Pugh (CTP) and BCLC class, AFP level, and tumor recurrence (Table 2).

Table 2. The association between *SOCS1* methylation with clinical parameters in the Italian cohort.

		***SOCS1* Methylation in HCC Tissues**			
		M (%)	**PM/UM (%)**	**All (%)**	***p***
Sex [F:M]		2 : 10 (17:83)	2 : 9 (18:82)	4 : 19 (17:83)	0.9999
Age [year, mean ± std]		70.9 ± 7.6	64 ± 6.9	67.6 ± 7.9	0.0177 *
Tumor size [cm, mean ± std]		4.9 ± 3.7	3.9 ± 2.1	4.4 ± 3.0	0.2399
AFP [median ng/mL, min–max]		7.4 (2–5094)	7.4 (3–139)	7.4 (2–5094)	0.1129
Etiology	HCV	3 (25)	6 (55)	9 (39)	0.2310
	HBV	2 (17)	0 (0)	2 (9)	
	Metabolic	6 (50)	3 (27)	9 (39)	
	no	1 (8)	2 (18)	3 (13)	
Histological grading	ES1	1 (9)	6 (60)	7 (33)	0.0465 *
	ES2	7 (64)	3 (30)	10 (48)	
	ES3-4	3 (27)	1 (10)	4 (19)	
CTP	A	10 (83)	8 (73)	18 (78)	0.5379
	B-C	2 (17)	3 (27)	5 (22)	
BCLC	0	8 (73)	10 (91)	18 (82)	0.5865
	1–2	3 (27)	1 (9)	4 (18)	
Recurrence (m)	<12 m	3 (75)	6 (43)	9 (50)	0.5765
	>12 m	1 (25)	8 (67)	9 (50)	

M: methylated; PM: partially methylated; UM: unmethylated; HCV: hepatitis C virus; HBV: hepatitis B virus; ES: Edmonson-Steiner; CPT: Child–Turcotte–Pugh; BCLC: Barcelona Clinic Liver Cancer. Statistical analysis: *$p \leq 0.05$ comparing M and PM/UM groups using Student's t-test for age, tumor size, and AFP level; using Chi-square test for etiology and histological grading; using Fisher's exact test for sex, CTP, BCLC stage, and recurrence.

4.1.2 Correlation of SOCS1 mRNA expression and DNA methylation

In parallel, *SOCS1* gene expression of similar sets of HCC specimens was performed by RT-qPCR. Primers for RT-qPCR were designed to cover the coding region in the CpG island 2 in

exon 2, as for DNA methylation. mRNA analysis showed no significant difference in *SOCS1* mRNA expression for non-tumoral, peritumoral, and tumoral tissues (Figure 3C).

To check whether *SOCS1* mRNA expression was correlated with *SOCS1* methylation as above, we performed a comparative analysis between two parameters in the data set. Our data showed that there was no association between *SOCS1* methylation and *SOCS1* expression (Figure 3D).

Additionally, protein blots shown in Figure 3E seemed to indicate a higher expression of DNMT1 and SOCS1 proteins in partially methylated tissue samples as compared to unmethylated samples. It might indicate the increased activity of DNMT1 and SOCS1 in these samples. However, due to a rather small number of protein samples available, this investigation was not completed.

4.1.3 Demethylation by 5-Azacytidine (5-AZA) in heterogenous in vitro models

Based on data in the clinical specimens showing frequent *SOCS1* methylation in tumoral tissues, we explored an epigenetic strategy to possibly induce the demethylation effect of *SOCS1* in HCC cells. However, since HCC is a very heterogeneous tumor, it is unclear whether the effect would be similar in different tumoral cells.

Here, we used six different cell lines representing cellular heterogeneity of HCC, as shown by their different phenotypes using flow cytometry (data from the FIF-HCC unit). As in the literature [153,154], the S2/progenitor subtypes HepG2 and Huh7, were identified with the presence of EpCAM+ cells where CD133+ cells were also noticed in Huh7. CSC markers CD24+ cells were present in HLE, HLF, and Huh7, CD13 cells in IHH, JHH6, Huh7, and HepG2, and CD90+ cells in IHH (Table 3).

Table 3. HCC cells phenotyping using several CSC markers (CD133, CD90, and CD24), a proposed epithelial CSC marker (EpCAM), and a dormant CSC marker (CD13).

Subtypes	Cell Line	EpCAM	CD133	CD90	CD24	CD13
Hepatocytes	IHH	-	-	+/–	-	+
S1/TGFβ-Wnt	HLE	-	-	+/–	+	-
	HLF	-	-	+/–	+	-
	JHH6	-	-	-	-	+/–
S2/progenitor	HepG2	+	-	-	-	+/–
	Huh7	+	+	-	+	+/–

+ : more than 90% of cells analyzed show the presence of cell surface marker; +/- : less than 10% of cells analyzed show the presence of cell surface marker.

5-Azacytidine (5-AZA) was chosen as a demethylating drug. First, we evaluated 5-AZA cytotoxicity by MTT test to determine the lethal concentration 50 (LC_{50}), ranging from 2 µM to 5 mM. Upon 5-AZA treatment for 24 h, the calculated LC_{50} was 128 µM for HLE, 14 µM for HepG2, 33 µM for HLF, 16 µM for Huh7, 41 µM for IHH, and 5 µM for JHH6 (Figure 4A).

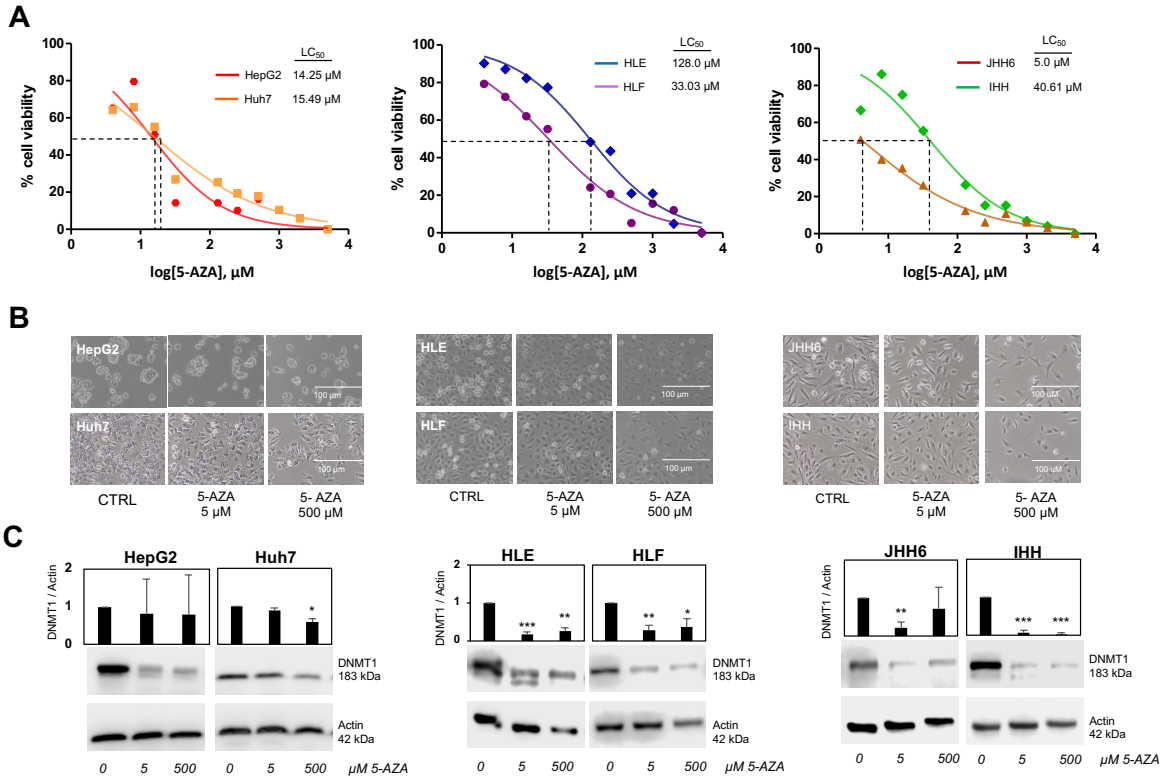


Figure 4. The effect of 5-Azacytidine (5-AZA) in *in vitro* models. (**A**) Dose-response of *in vitro* models: S2/progenitor subtypes: HepG2 and Huh7; S1/TGFβ-Wnt subtypes: HLE, HLF, and JHH6, and immortalized hepatocytes IHH. All cells were treated with 2 µM to 5 mM of 5-AZA for 24 h and cytotoxicity assay was performed by MTT test. Dashed lines show LC_{50} value for each cell line. (**B**) Cellular morphology of *in vitro*

models after 24 h treatment of 5 µM and 500 µM of 5-AZA. (**C**) Quantitative graphs and representative blots of DNMT1 protein expression (183 kDa) after 24 h treatment of 5 µM and 500 µM of 5-AZA. Actin (42 kDa) was used as a housekeeping protein. Graphs presented as mean ± SD calculated from at least three independent experiments. Statistical analysis for data in Figure 4C: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ using Student's t-test relative to CTRL (0 µM) in each cell line. MTT: 3(4,5-dimethyl thiazolyl-2)-2,5 diphenyltetrazolium.

Since the cytotoxicity evaluation of 5-AZA on all cells revealed a minimum LC_{50} value of 5 µM we chose to further evaluate this concentration. Morphological analysis showed that 5-AZA of 5 µM did not alter the morphology of the cells. To compare, we treated the cells with a 100X higher concentration of 5-AZA (500 µM), which resulted to change in the cellular morphology of the cells. (Figure 4B). Both concentrations reduced the expression of DNMT1 protein to different extents, which seemed to be correlated with cellular subtypes. The lowest DNMT1 reduction after 5 µM treatment was noticed in non-tumoral cells IHH reaching up to a 93% decrease ($p < 0.001$). The S1/TGFβ-Wnt subtypes, HLE, HLF, and JHH6 showed reductions of around 80% (83%, 73%, and 79%, respectively, $p < 0.01$). On the other hand, the S2/progenitor subtypes HepG2 and Huh7 showed rather slight decreases upon 5-AZA treatments (17% and 10%, respectively). A significant reduction was noticed only following 500 µM treatment in Huh7 of around 40% (Figure 4C).

4.1.4 SOCS1 modulation following demethylation by 5-Azacytidine (5-AZA) treatment

First, we checked the *DNMT1* and *SOCS1* gene expressions among cell lines. We noticed that, compared to non-tumoral IHH cells, the *DNMT1* expressions (Figure 5A) in HCC cells were significantly higher for more than 2-fold ($p < 0.05$). For *SOCS1*, expression in JHH6 was comparable to that of IHH, while it was significantly higher in HLE and HLF for around 4-fold, and 6-fold, respectively ($p < 0.05$). *SOCS1* expression was noticeably much higher in S2/progenitor subtypes Huh7 and HepG2, accounting for around 60-fold for both cells ($p < 0.05$) (Figure 5B).

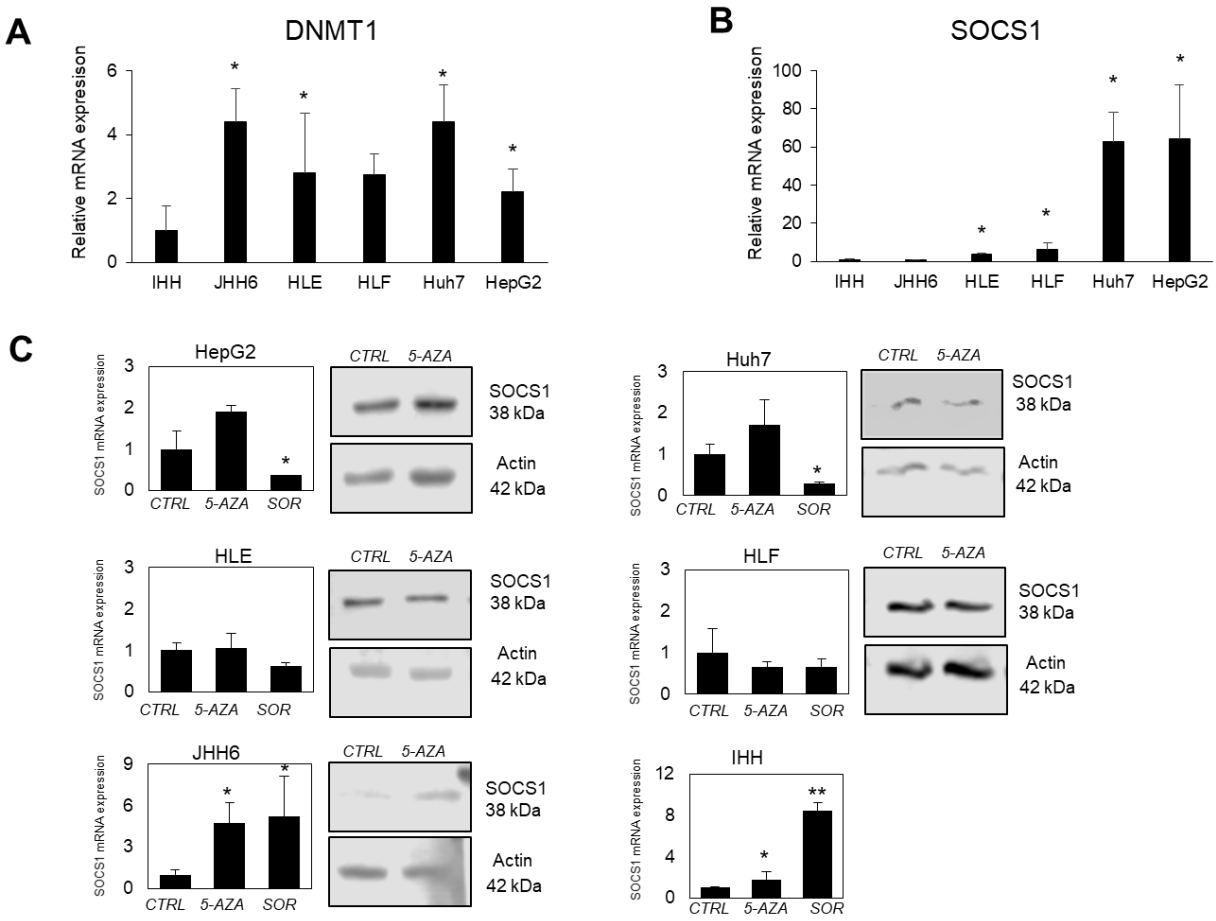


Figure 5. The effect of 5-Azacytidine (5-AZA) in *SOCS1* expression in *in vitro* models. (**A**) Relative mRNA expression of *DNMT1* in HCC cell lines JHH6, HLE, HLF, Huh7, and HepG2 compared to non-tumoral IHH cells (= 1.0) (**B**) Relative mRNA expression of *SOCS1* in HCC cell lines compared to non-tumoral IHH cells (= 1.0). Statistical analysis (Figure 5A-5B): *$p < 0.05$ using Student's t-test relative to IHH. (**C**) mRNA expression and representative protein blot of *SOCS1* (38 kDa) in hepatic cell lines after 24 h treatment of 5 μM of 5-AZA and 50 μM of sorafenib. Actin (42 kDa) was used as housekeeping in the protein blot. Statistical analysis (Figure 5C): *$p < 0.05$; ** $p < 0.01$ using Student's t-test relative to CTRL for each cell line. Graphs presented as mean ± SD calculated from at least three independent experiments.

The 5-AZA non-toxic concentration of 5 μM was used to investigate *SOCS1* modulation in these cells. As shown in Figure 5C, following the treatment of 5 μM of 5-AZA for 24 h, the *SOCS1* mRNA expression was increased to different extents. HCC cell lines belonging to the S2/progenitor subtypes Huh7 and HepG2 (HepG2 and Huh7) gained an approximately 2-fold increase in SOCS1 mRNA expression after 5-AZA treatment, whereas the *SOCS1* expression in S1/TGFβ-Wnt subtypes HLE and HLF was unchanged. However, a significant increase of around 4-fold was noticed in JHH6 cells ($p < 0.05$). The non-tumoral cells IHH showed a 2-fold increase in *SOCS1* expression ($p < 0.05$).

We also compared 5-AZA with sorafenib treatment. Sorafenib is an approved molecular targeted therapy against VEGFR and Raf-kinases for HCC. The concentration used for this drug relating to this task was based on the results of the cytotoxicity of sorafenib, described in Task 2. Upon 24 h of 50 μM sorafenib, in contrast to 5-AZA, the SOCS1 expression was decreased for HepG2 and Huh7 ($p < 0.05$). Sorafenib treatment was able to increase the expression of SOCS1 only in JHH6 and IHH cells ($p < 0.05$ and $p < 0.01$, respectively).

The result of this task is published in Diagnostics in October 2021: Cabral, L.K.D.; Reyes, P.A.C.; Crocè, L.S.; Tiribelli, C.; Sukowati, C.H.C. The Relevance of SOCS1 Methylation and Epigenetic Therapy in Diverse Cell Populations of Hepatocellular Carcinoma. Diagnostics 2021, 11, 1825. https://doi.org/10.3390/diagnostics11101825

4.2 TASK 2: Identification of potential molecular targets of therapy through the utilization of bioinformatic analysis in *in vitro* models of heterogeneity

Clinical and histopathologic evidence describe HCC as a heterogeneous disease, but there is still a need to provide a coherent molecular explanation for HCC heterogeneity [39]. Several researchers have utilized -omics approaches to classify HCC, focusing on their molecular and cellular taxonomies [38,39,155]. These classifications resulted in the so-called molecular classes/subtypes that reflect the heterogeneity of the cells. Each class/group/subtype shows distinct cellular phenotypes, disactivations of molecular pathways, differentiation, and sensitivities to given treatments. In TASK 2, we aimed to look at potential targets for HCC treatment, taking advantage of reported molecular classifications together with bioinformatics tools. The exploration of the validity of proposed targets for the treatment of HCC was assessed in experimental models comprising different cellular classifications.

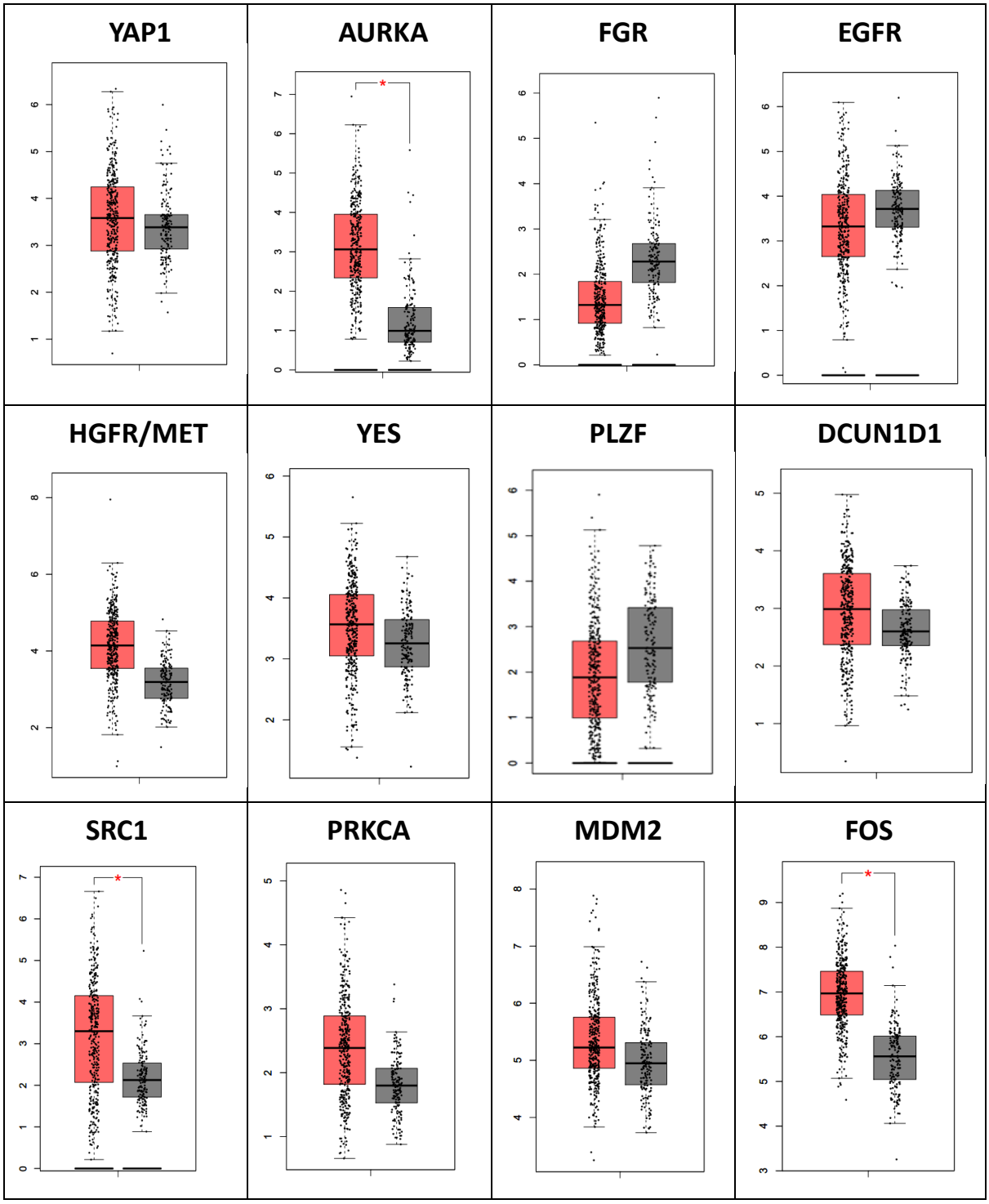
4.2.1. Identification of candidate targets

We employed an *in-silico* strategy to consider the innate heterogeneity of HCC by gradual filtering, to discover potential drug targets that may comprise cellular heterogeneity (Figure 2). From the protein-protein interaction (PPI) networks (Appendix), we identified 982 and 3659 common proteins from Hoshida and Boyault extended classifications, respectively. Gradual selection from those proteins, by excluding housekeeping genes and including proto-

oncogenes, resulted in 26 proto-oncogene targets. From those targets, following GEPIA analysis on their clinical distributions and associations according to TCGA and GTEx datasets (Figure 6) (comprising 369 liver cancer tissues vs. 160 normal tissues), we further narrowed down the targets to 16 candidates as shown in Table 4.

Table 4. List of 16 proto-oncogene targets and their respective UniProt ID, protein name, gene symbol, and gene name.

UniProt ID	Protein Name	Gene	Gene Name
P46937	Transcriptional coactivator YAP1	YAP1	yes-associated protein 1
O14965	Aurora kinase A	AURKA	aurora kinase A
P09769	Tyrosine-protein kinase Fgr	FGR	FGR proto-oncogene
P00533	Epidermal growth factor receptor	EGFR	epidermal growth factor receptor
P08581	Hepatocyte growth factor receptor	HGFR, MET	MET proto-oncogene, receptor tyrosine kinase
P07947	Tyrosine-protein kinase Yes	YES1	YES proto-oncogene 1, Src family tyrosine kinase
Q05516	Zinc finger and BTB domain containing 16	PLZF, ZBTB16	zinc finger and BTB domain containing 16
Q96GG9	DCN1-like protein 1	DCUN1D1	defective in cullin neddylation 1 domain containing 1
P12931	Proto-oncogene tyrosine-protein kinase Src	SRC1, ASV	SRC proto-oncogene, non-receptor tyrosine kinase
P17252	Protein kinase C alpha type	PRKCA	protein kinase C alpha
Q00987	E3 ubiquitin-protein ligase Mdm2	MDM2	MDM2 proto-oncogene
P01100	Protein c-Fos	FOS	Fos proto-oncogene, AP-1 transcription factor subunit
P22681	E3 ubiquitin-protein ligase CBL	CBL	Cbl proto-oncogene
P06241	Tyrosine-protein kinase Fyn	FYN	FYN proto-oncogene, Src family tyrosine kinase
P05412	Transcription factor Jun	JUN	Jun proto-oncogene, AP-1 transcription factor subunit
P42566	Epidermal growth factor receptor substrate 15	EPS15	epidermal growth factor receptor pathway substrate 15


YAP1
AURKA
FGR
EGFR
HGFR/MET
YES
PLZF
DCUN1D1
SRC1
PRKCA
MDM2
FOS

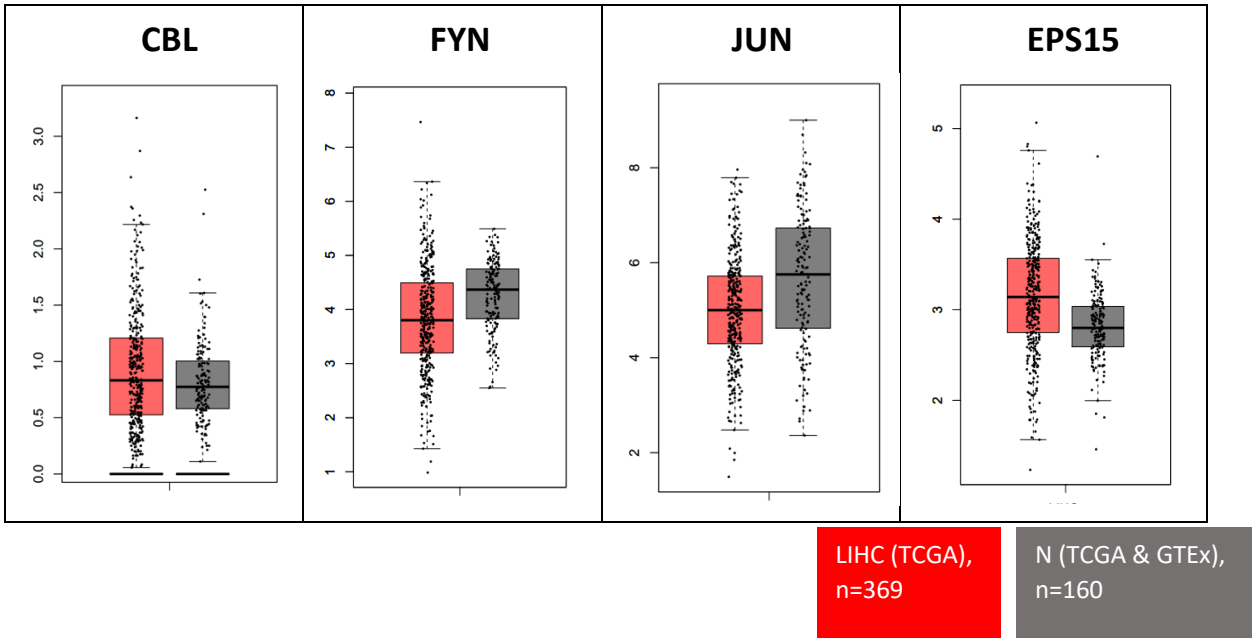


Figure 6. GEPIA analysis of the 16 potential proto-oncogene targets. Data show tissue gene expression in TCGA Data Liver hepatocellular carcinoma (LIHC) = 369 (in red) vs. TCGA and GTex Data Normal = 160 (in gray). Data presented as expression -log2(TPM +1) transformed for differential analysis. Statistical analysis: * indicates differentially expressed genes according to the selected data set (TCGA tumors vs TCGA normal + GTEx normal, using one-way ANOVA. Genes with higher log_2FC values (defined as median(Tumor) - median(Normal) and lower p values than pre-set thresholds (log_2FC = 1 and *p*-value = 0.01) are considered differentially expressed genes.

4.2.2. Expression of targets in in vitro, in vivo, and clinical models

We then analyzed the baseline expression levels of the 16 targets (Table 4) in the *in vitro* models and compared expressions in HCC cells to those in IHH. We observed that in HCC cells 10 out of the 16 (62%) proto-oncogene target genes were up-regulated and 6 were downregulated in the HCC cell lines (Figure 7A). Further comparison of gene expressions separating the two cell subtype groups showed that 13/16 proto-oncogene targets (81%) were up-regulated in the S2/progenitor subtype, whereas only 10/16 (62%) were up-regulated in the S1/TGFβ-Wnt subtype (Figure 7B).

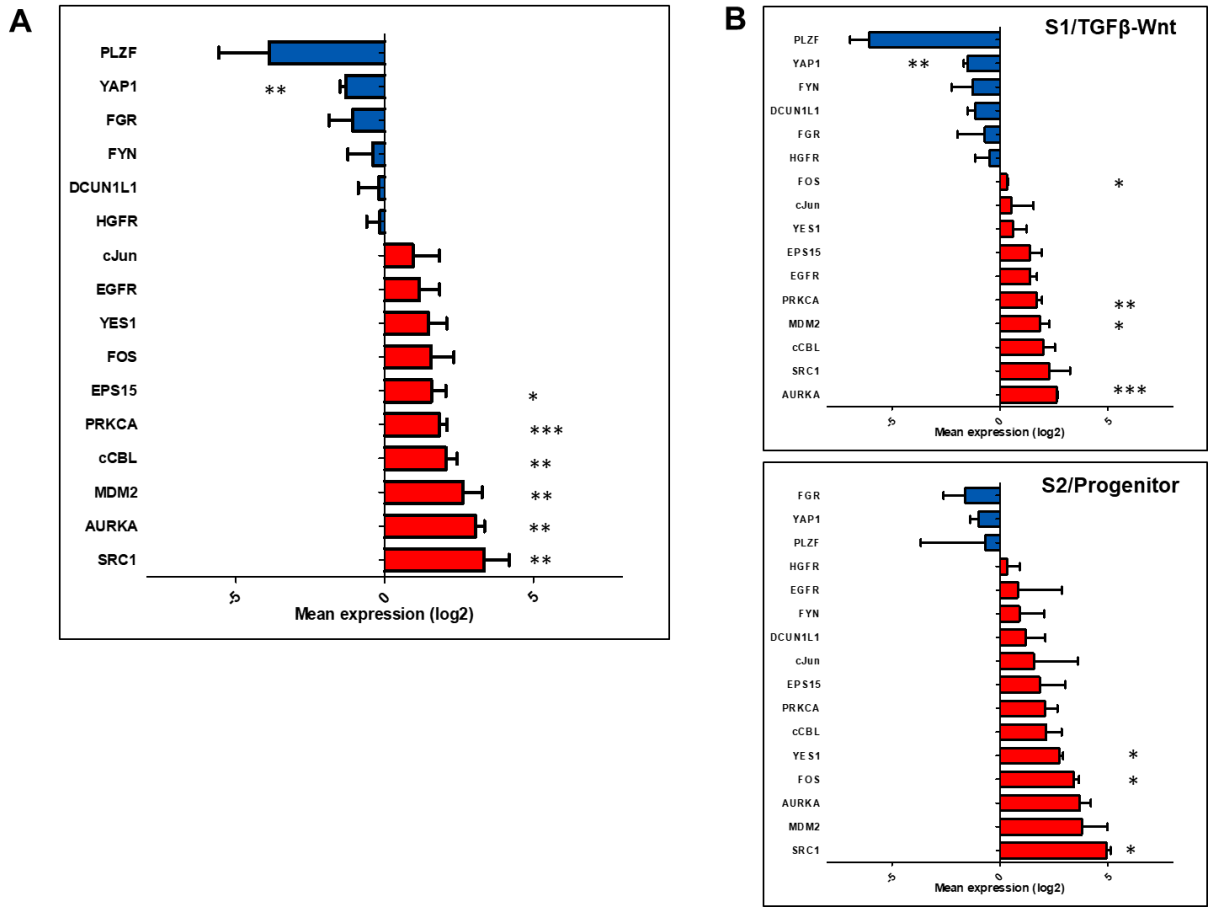


Figure 7. Baseline mRNA expression of targets in the various HCC cell populations. (**A**) Distribution of targets showing the upregulated and the downregulated proto-oncogenes. (**B**) Distribution of relative expression of candidate targets between the S1 and S2 cell populations. Data are presented as the mean expression values (log2) from three independent samples of each cell line. Statistical analysis for data in Figure 7A and 7B: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ using the one-sample t-test vs. mean expression of immortalized hepatocytes IHH as 0.00. Red - up-regulated, Blue - down-regulated.

Assessing the result of the proto-oncogene expression in the HCC cell lines, we identified the most upregulated (*SRC1*) and most downregulated (*PLZF*) targets. We further investigated their distributions in the HCC samples of the Italian and Vietnamese cohorts (Figure 8). Our data showed that in Italian HCC specimens, the mRNA distribution for *SRC1* seemingly displays an increasing trend in the mRNA expression of non-tumoral, to peri-HCC and HCC tissues. Median expression values were 0.34 (0.14-0.89), 0.84 (0.34-1.46), and 1.00 (0.17-2.40), in distal, peri-HCC and HCC tissues, respectively. Meanwhile, the Vietnamese cohort showed no difference or trend in median mRNA expression of *SRC1* between non-tumoral, peri-HCC, and HCC samples. However, analysis of the Vietnamese cohort looking at *PLZF* mRNA expression displays a seemingly regressive trend. Median values were 10.40 (6.02-13.63), 5.18 (2.81-10.44), and 2.26 (0.40-8.74), in non-tumoral, peri-HCC and HCC tissues,

respectively. Meanwhile, there was no difference between PLZF expressions across non-tumoral, peri-HCC, and HCC tissues for the Italian cohort. Values correspond to median mRNA expression (Q1–Q3). However, statistical analysis of these data using, one-way ANOVA did not indicate a significant value, indicating only a pattern but not an absolute test of the difference between generated results.

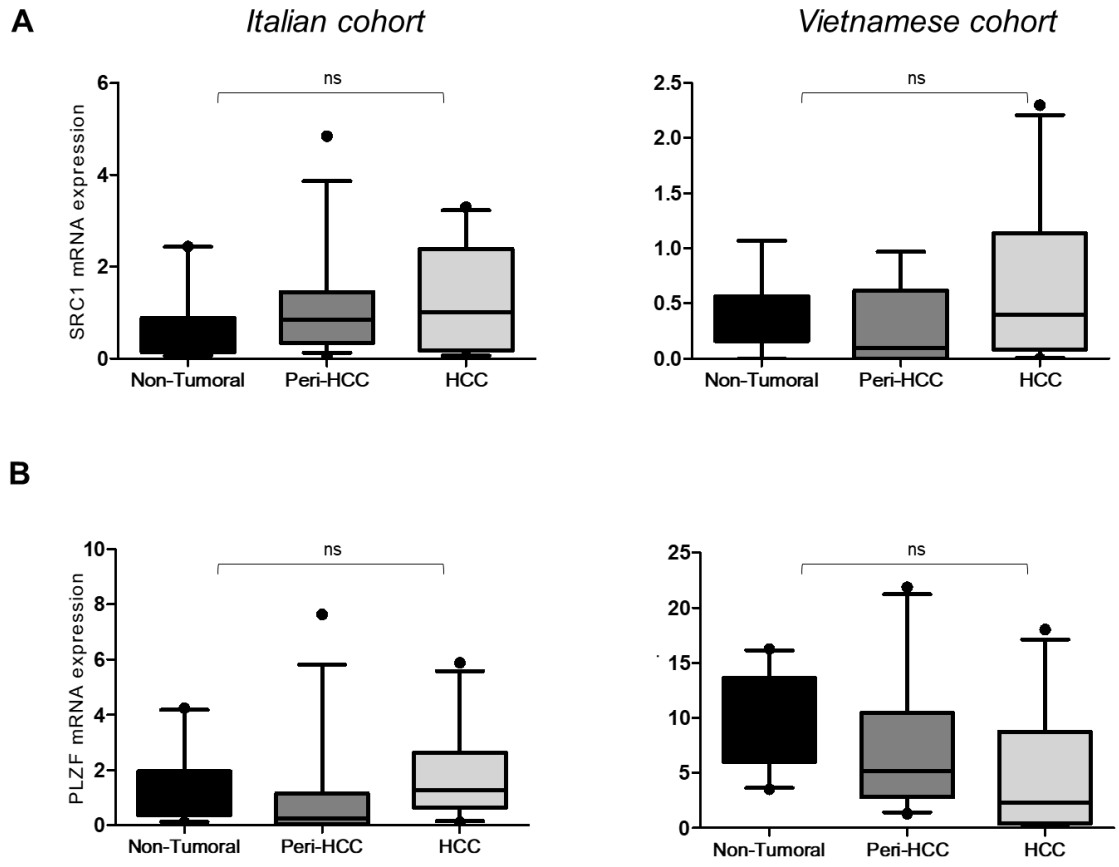


Figure 8. Distribution of *SRC1* and *PLZF* in HCC clinical samples. (**A**) mRNA distribution of *SRC1* in Italian and Vietnamese cohorts. (**B**) mRNA distribution of *PLZF* in Italian and Vietnamese cohorts. Statistical analysis for data in Figure 8A and 8B: ns, using non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test to compare data of non-tumoral, peri-HCC, and HCC tissues. ns: not significant

We also tried to understand how these two targets are distributed in liver tissue from mouse samples reflecting different stages of liver injury (data from the FIF-HCC unit). Noticeably, there was an upregulated expression of *Src1/pp60c-src* for 2 to 5-fold higher, in HBV-TG compared to WT ($p < 0.0001$). Paired analysis within similar age groups confirmed the observed differences. Interestingly, the expression level of *Src1/pp60c-src* in HBV-TG liver tissues sequentially increased with the progression of liver damage (Figure 9A). Meanwhile, for *Zbtb16/PLZF* there was an observed regressive decrease in the mRNA expression in mice,

but is seemingly correlated to age since the decrease in *Zbtb16/PLZF* mRNA is both observed in the WT and TG groups in a more or less similar extent (Figure 9B).

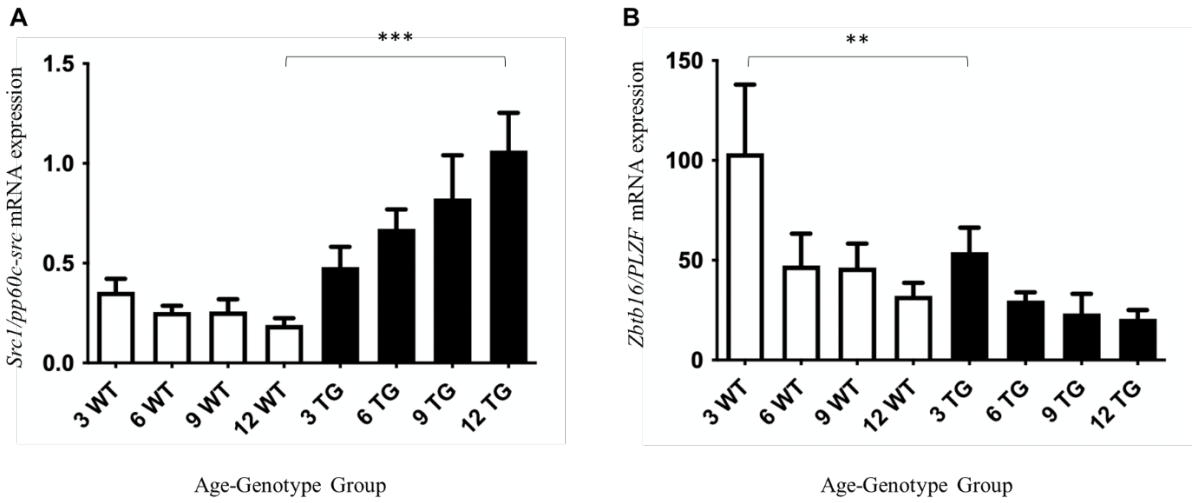


Figure 9. Distribution of *Src1/pp60c-src* and *Zbtb16/PLZF* in liver tissues of HBV-TG and WT mouse models. Bar graphs represent mean mRNA expression in the different stages of liver injury of HBV-TG and WT counterparts. Statistical analysis for data in Figure 9A and 9B: ** $p \leq 0.01$; *** $p \leq 0.001$, using one-way ANOVA followed by Newmans-Keul multiple comparison test revealing a significant difference between *Src1/pp60c-src* mRNA expressions of 12 months WT and TG mice and *Zbtb16/PLZF* mRNA expressions of 3 months WT and TG mice. 3 WT: 3 months wildtype mouse; 3 TG: 3 months HBV-transgenic mouse. The number before the genotype indicates the age of the mouse model. WT: wild-type; TG: HBV-transgenic.

4.2.3. Effect of targeted treatments on different cell populations

We used three different treatment strategies in the different HCC cell populations. As shown previously in TASK 1, the LC_{50} of 5-AZA was 128 μM for HLE, 33 μM for HLF, 41 μM for IHH, 16 μM for Huh7, 14 μM for HepG2, and 5 μM for JHH6. In TASK 2, we chose the concentration of 5 μM as a non-lethal concentration for 5-AZA epigenetic therapy. This concentration was able to inhibit the methylation activities of DNMT1 allowing the reversal of transcriptional silencing, as seen in our data in TASK 1.

For the SOR, the following LC_{50} values shown in Figure 10A were calculated after 24 h of exposure of the cells to the drug. Cells belonging to the S2/progenitor subtype appear to be more sensitive to SOR as compared to cells belonging to S1/TGFβ-Wnt subtype. Noticeable morphological changes were observed in HLE, HLF, and JHH6 cells after treatment with 50 μM SOR (Figure 10B).

PD-L1 silencing and gene knockdown by siRNA resulted in a decrease of mRNA expression in all cell populations after 48 h of exposure to 20 nM of siR-PD-L1. Following RNA silencing,

the extent of PD-L1 mRNA reduction was 70% and 64% for Huh7 and HepG2, respectively ($p < 0.05$). Higher extents of downregulation were noticed in the S1/TGFβ-Wnt subtype cells, for 70%, 82%, and 91% for HLE, HLF, and JHH6, respectively ($p < 0.05$). PD-L1 downregulation was also noticed for IHH cells for around 80% ($p < 0.001$) (Figure 10C).

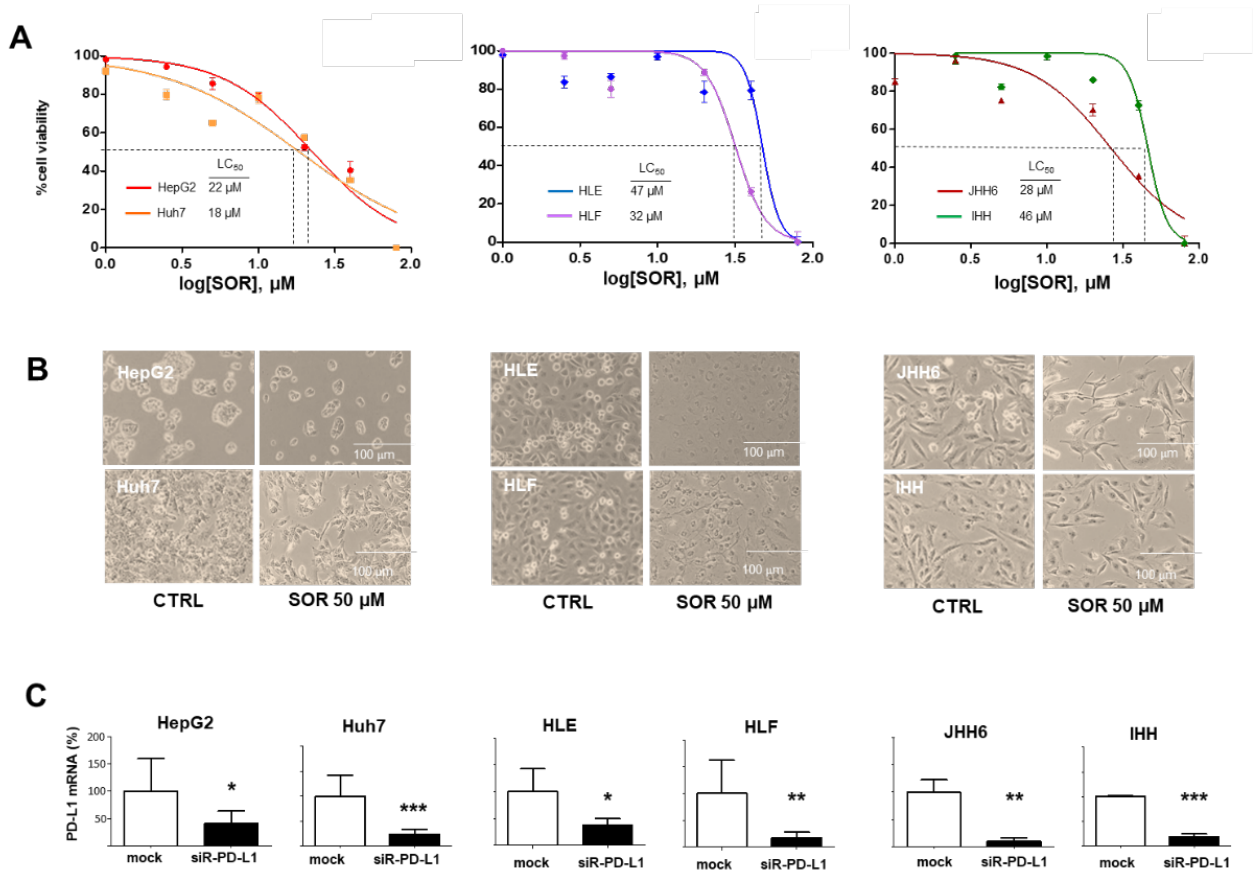


Figure 10. Sorafenib (SOR) treatments and PD-L1 mRNA silencing (siR-PD-L1) in *in vitro* models. (**A**) Cell viability upon 24 h treatment with 1 µM to 80 µM of SOR. Dashed lines show the value of LC_{50}. (**B**) Cell morphology after 24 h treatment of 50 µM of SOR. (**C**) Downregulation of PD-L1 mRNA expression after 48 h of 20 nM PD-L1 silencing. Statistical analysis for data in Figure 10C: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ using Student's t-test (vs. mock) in each cell line. Graphs presented as mean ± SD calculated from at least three independent experiments. SOR: sorafenib, siR-PD-L1: PD-L1 silencing.

4.2.4. Effect of treatments on the dysregulations of proto-oncogene targets

From the results of the MTT assays (5-AZA and SOR) and silencing experiments (siR-PD-L1), we further evaluated the dysregulations of the 16 proto-oncogene targets in Table 4 in the different cell populations. For the concentration of the treatments, concentrations of 5 µM and 50 µM were selected for 5-AZA and SOR, respectively. For the silencing, the treatment with

20 nM of siR-PD-L1 was able to significantly reduce PD-L1 mRNA expression in all cell lines investigated. Figure 11 shows a representative heat map indicating the dysregulated mRNA expression of the proto-oncogenes.

Among the three treatment modalities, 5 µM of 5-AZA did not show significant downregulation effects on proto-oncogene targets in the different cells, except for *FGR* and *PLZF*. For SOR treatment, there were downregulation effects in proto-oncogenes *FGR*, *PLZF*, and FOS. Interestingly, we also observed that the 50 µM SOR treatment downregulated proto-oncogene mRNA expression mostly in the cells belonging to the S2/progenitor subtype.

Notably, for the immune-targeting treatment results, using 20 nM siR-PD-L1 showed effective downregulation in almost all proto-oncogenes in all cell lines evaluated.

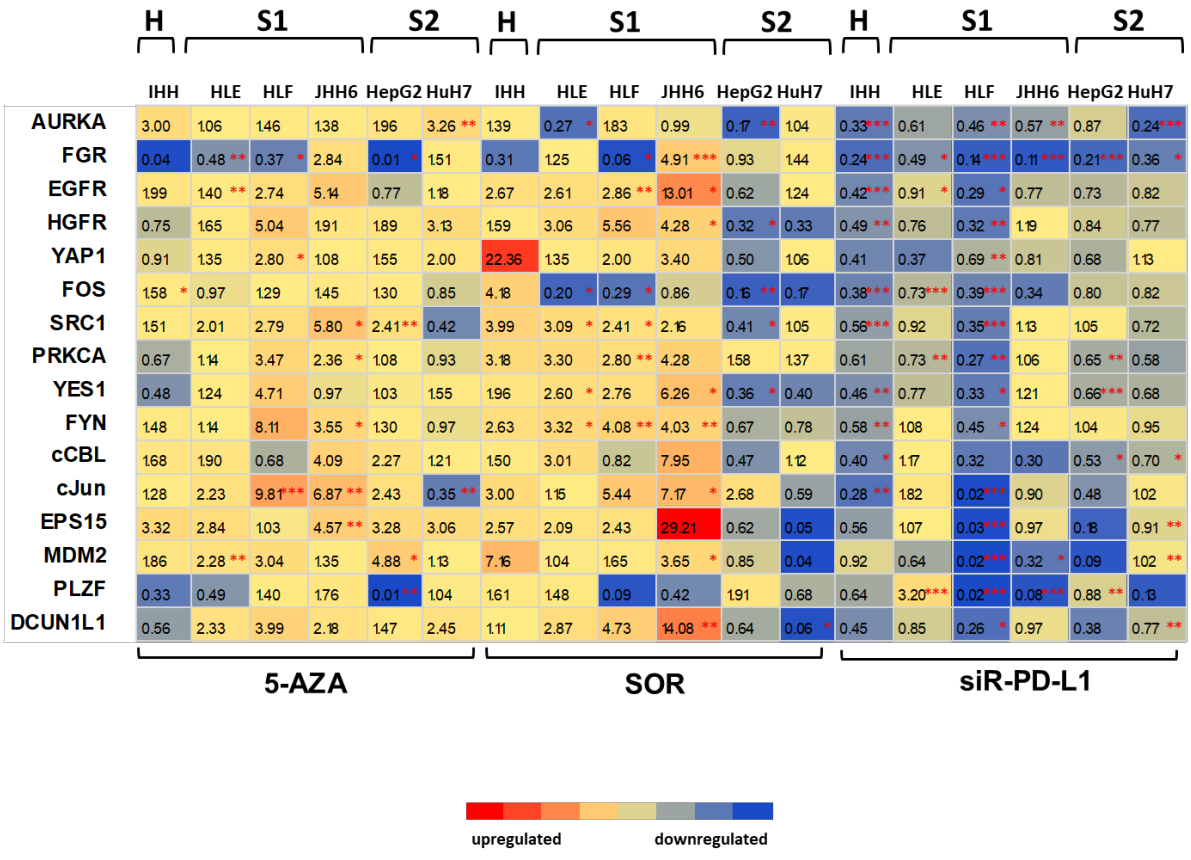


Figure 11. Dysregulation of mRNA expressions of proto-oncogene targets in liver cancer cell lines after various treatments. The heat map indicates the up-regulation and downregulation of the markers after treatment with 5 µM 5-AZA, 50 µM SOR, and 20 nM siR-PD-L1. Data presented as mean calculated from at least three independent experiments. Statistical analysis for data in Figure 11: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ using Student's t-test evaluating the difference between means of treated vs untreated cells.

The result of this task is published in Biomedicines in January 2023: Cabral, L.K.D.; Giraudi, P.J.; Giannelli, G.; Dituri, F.; Negro, R.; Tiribelli, C.; Sukowati, C.H.C. Network Analysis for the Discovery of Common Oncogenic Biomarkers in Liver Cancer Experimental Models. Biomedicines 2023, 11, 342. https://doi.org/10.3390/biomedicines11020342

4.3 TASK 3: Characterization of unique targets of therapy and evaluate their anti-cancer effects as monotherapy or combination treatment

Despite numerous studies for early diagnosis, prognostic value, and treatment, HCC remains one of the most difficult to cure end-stage liver diseases [79]. The carcinogenesis complexity makes it hard to diagnose while the heterogeneity (tumor extent, patient comorbidities, and severity of liver dysfunction) challenges both management and treatment [18]. Radical treatments such as surgical resection and liver transplant are considered only for early-stage HCC [156] and do not apply to more than two-thirds of HCC cases as advanced and metastatic stages [157]. For unresectable HCC, options of radiofrequency ablation (RFA) and transarterial chemoembolization (TACE) can be helpful [156] but still offers minimal improvement in patient overall survival. To date, identifying a highly effective systemic therapy for HCC has remained a challenge to its management. In TASK 3, we aimed to identify unique targets for therapy that could provide anti-cancer treatment effects comprising heterogeneity.

4.3.1. Identification of unique targets for therapy

We further analyzed the proto-oncogene targets from TASK 2. Bioinformatic tools were used to understand the functions, roles, and associations of the 16 proto-oncogenes. The association of the target molecules was further classified using the STRING database [159]. The result of the protein-protein network considered physical and functional associations of the proteins of interest. This analysis indicated a PPI enrichment p-value of 4.17e-10, which indicates that the 16 proteins are at least partially biologically connected, as a group (Figure 12).

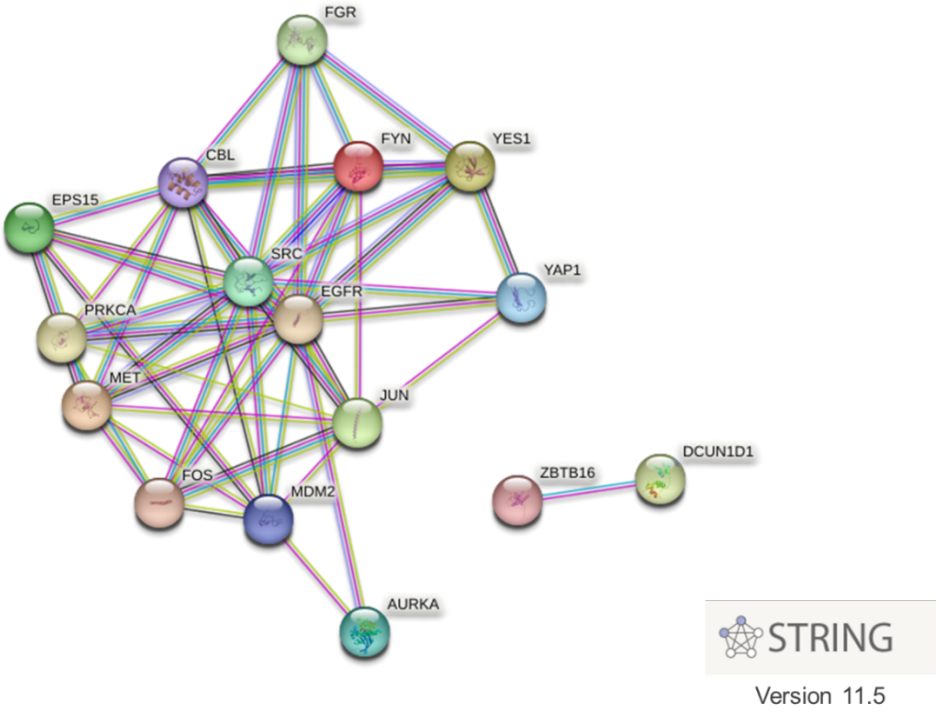


Figure 12. Enrichment network interaction of the 16 proto-oncogene targets.

Using the g.profiler tool [160] we performed a gene enrichment analysis of the protein targets and were able to identify a unique group of non-membrane tyrosine kinases belonging to the Src family of tyrosine kinases (SFKs) (Figure 13). Four out of the nine members of this family of kinases, *SRC1*, *FGR*, *YES1*, and *FYN,* were included in our list of potential targets. This directed our focus on this rather unique clustering in our network of proteins.

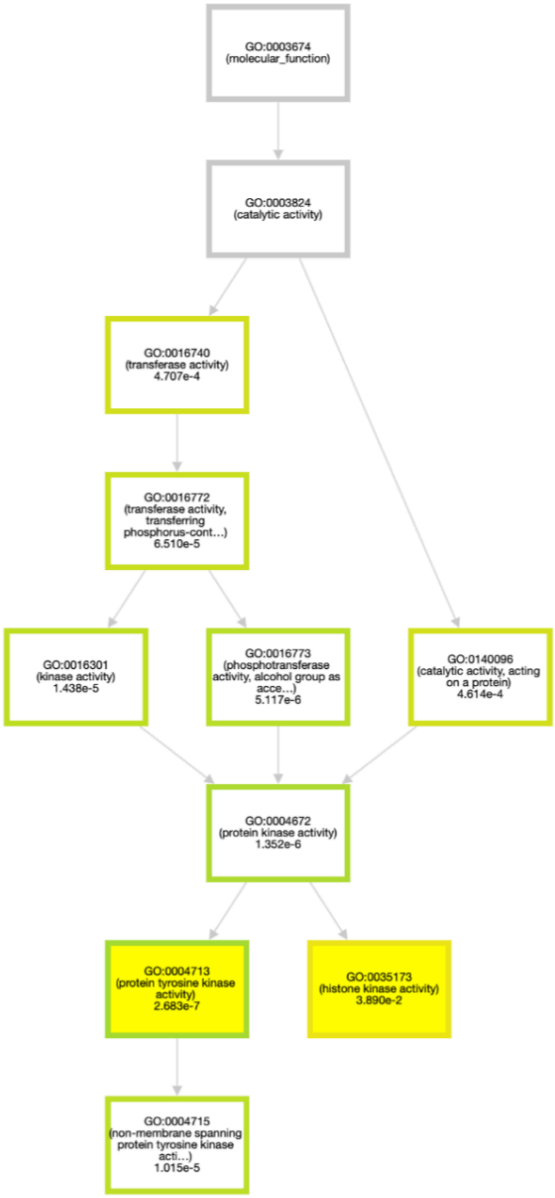


Figure 13. Gene enrichment analysis result of the 16 proto-oncogene targets.

4.3.2 Dysregulation effects of various cancer treatments on SRC protein expression

Referring to the data in Figure 11 of TASK 2, we further looked into the dysregulation effects of the three treatments on SFKs. Among the four members of the SFKs, the FGR mRNA was downregulated in at least three cell lines following all treatments. 5-AZA was able to reduce *FGR* mRNA expression, ranging between 52% and 99% ($p < 0.05$) in four cell lines. SOR reduced its expression, ranging between 10% and 94% in two cell lines. Notably, sir-PD-L1-treated cells showed a significant reduction of *FGR* expression in all six cells lines, ranging

between 51% and 89% ($p < 0.001$ for IHH, HLF, JHH6, and HepG2; $p < 0.05$ for HLE and Huh7) (Figure 11). While for the other SFKs, the decrease in expressions after treatments varied.

Western blot analysis was done to understand the effect of treatments on the SFKs. The c-Src antibody used is recommended for the detection of c-Src, Yes p62, Fyn p59, c-Fgr p55, and c-Src-2 (50 kDa). As shown in Figure 14, the three treatment modalities mostly reduced the c-Src protein expressions in the cell lines investigated, except for PD-L1-silenced HepG2 cells, 5-AZA and SOR treated Huh7 and JHH6 cells, and 5-AZA treated IHH cells. Evaluating the mock and siR-PD-L1 cells, the decrease in c-Src protein expression is reduced in 5 of the 6 cell lines but is only significantly reduced in the HLE and HLF cell lines ($p < 0.05$). For HepG2 cells the c-Src reduction was observed only in the cells treated with 5-AZA and SOR ($p < 0.05$).

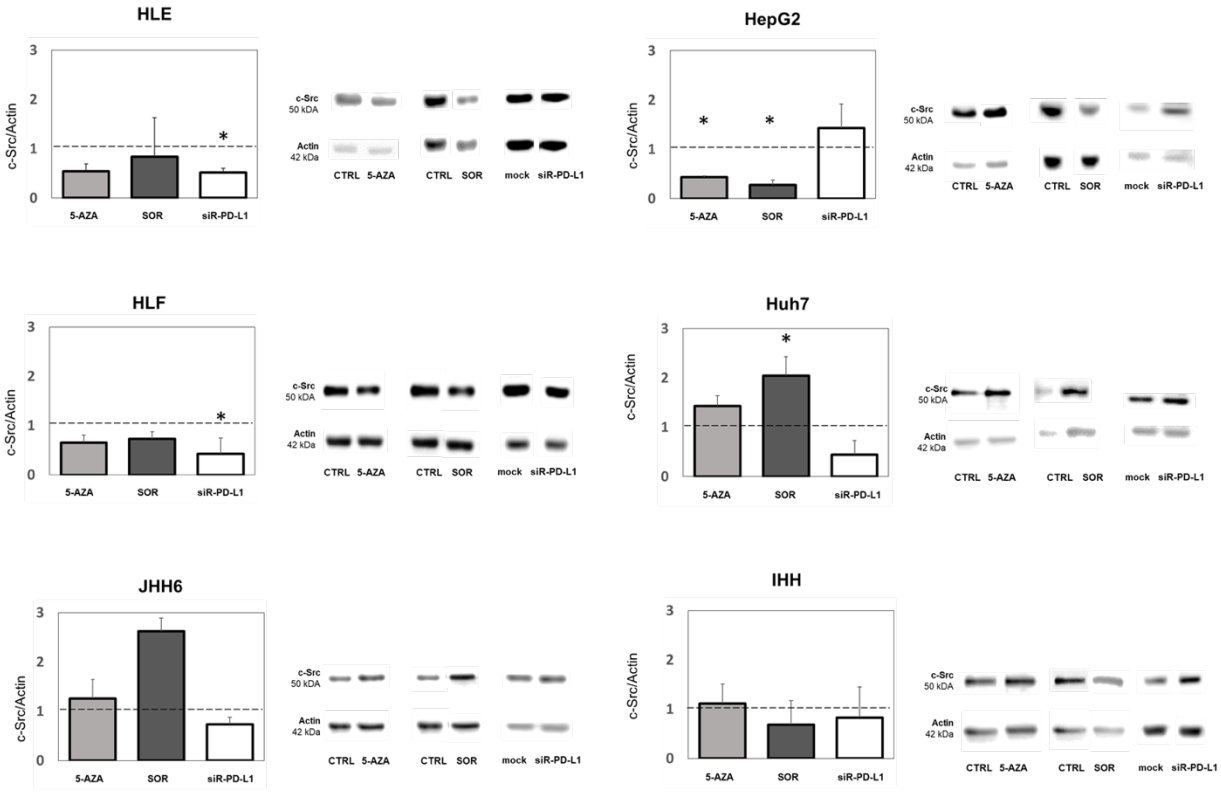


Figure 14. Effect of various treatment modalities on c-Src protein expression. Bar graphs indicate the mean protein expression of three independent experiments. Representative immunoblots indicate c-Src and actin expression among different HCC cell lines after treatment with 5-AZA, SOR, and siR-PD-L1. Statistical analysis for data in Figure 14: * $p \leq 0.05$ using Student's t-test against CTRL or mock (=1.00; shown as dashed line). Actin (42 kDa) was used as a housekeeping protein against the c-Src (50 kDa). Graphs presented as mean ± SD calculated from at least three independent experiments.

4.3.3 Effect of Src family of tyrosine kinases (SFK) inhibitors to various in vitro models as monotherapy vs combined therapy with sorafenib (SOR)

In TASK 2, referring to Figure 7A we were able to assess the baseline expression of the SFKs in the different cellular models. Gene expression analysis revealed that in HCC cells, *SRC1* and *YES* were 10 and 3-fold higher ($p < 0.05$), respectively, while *FYN* and *FGR* were downregulated (1.5 and 2-fold less, respectively) compared to IHH.

We then assessed the cytotoxicity effect of two SFK inhibitors saracatinib (SAR) and dasatinib (DAS) (Figure 15). These inhibitors were chosen because they have preferential inhibitory effects, whereas DAS has inhibition specificity, although quite low on potential targets including Lck, Fyn, and Yes1 [161]. Meanwhile, SAR is highly selective for non-receptor tyrosine kinases, including Src1, Yes1, Lck, and Abl [162]. Clinical reports suggest that DAS and SAR are tolerated to different extents, with SAR providing more manageable adverse effects [163].

Following *in vitro* treatments after 24h, both SAR and DAS treatment alone was not significantly toxic to the cells. A similar effect was noticed for 10 μM SOR treatment alone, showing a modest cell viability reduction between 70 and 88% in HepG2, Huh7, HLE, and IHH cells while it was not changed for HLF and JHH6. Interestingly, combination therapy between these SFK inhibitors with SOR resulted in a dose-dependent response to the drugs (Figure 15).

HCC cell lines HepG2 and HLE were the most sensitive to combined treatment of 0.02 to 5.0 μM SFK inhibitors + 10 μM SOR, with LC_{50} values for HepG2 and HLE of 0.06 and 0.6 μM for SAR-SOR and 0.01 and 0.02 μM for DAS-SOR, respectively. For HLF, JHH6, and Huh7 the LC_{50} values were 5.7, 4.5, and 2.6 μM respectively for SAR-SOR, while for DAS-SOR, their LC_{50} values were 1.8, 4.6, and 1.2 μM, respectively. It is important to notice that in IHH control cells, the effect of the SAR-SOR combination was observed to be less toxic compared to DAS-SOR treatment, with LC_{50} of 4.8 vs 0.1 μM, respectively.

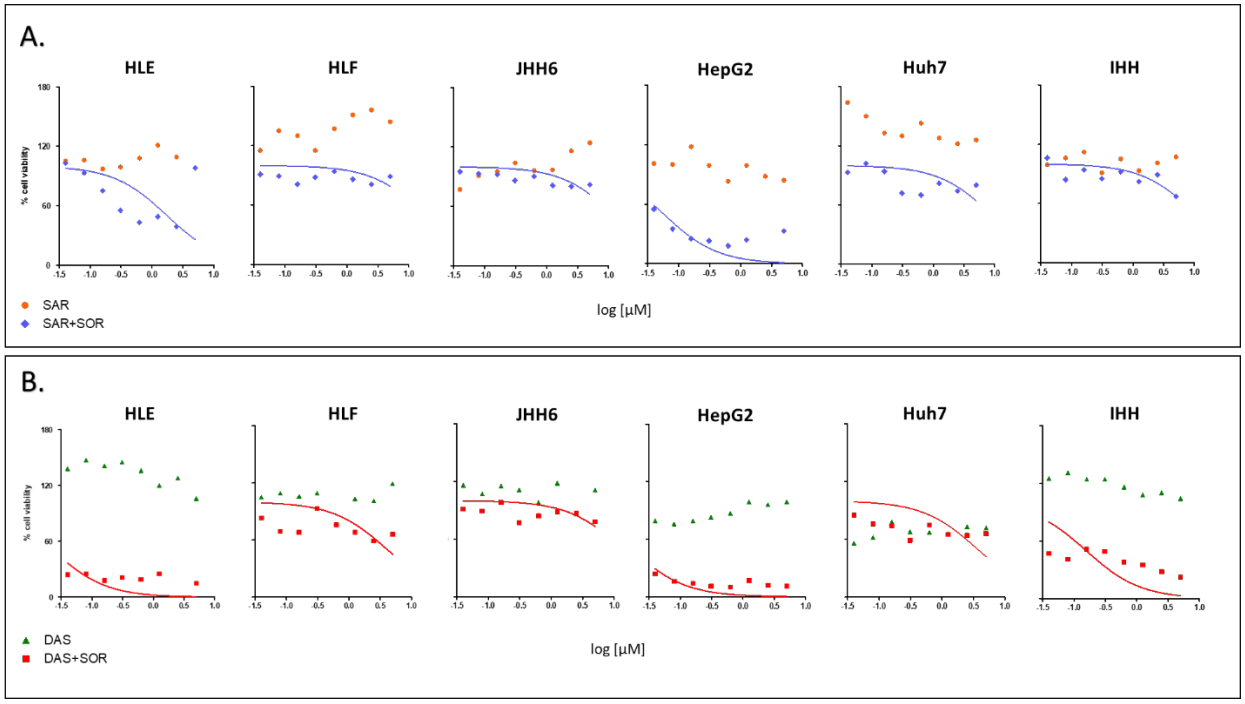


Figure 15. Cytotoxic evaluation on the effect of sorafenib (SOR) and Src family of tyrosine kinases (SFK) inhibitors on cell lines. (**A**) SAR and (**B**) DAS as mono vs combined treatment (+ SOR). Single dots/points represent the % viability of cells for each concentration of drug treatment; the solid line represents the dose-response curve based on the non-linear regression analysis. Data is calculated as mean from at least three independent experiments.

We further evaluated the anti-migration effect of the SFK inhibitors as mono and combined therapy utilizing the wound scratch healing assay. Wound closure was measured as the remaining area uncovered by the cells in 0, 24, 48, and 72hrs (Figure 16). The combined treatment of SAR+SOR or DAS+SOR revealed a wider wound area compared to SFK inhibitor or SOR treatment alone, indicating better inhibition ability of combination treatments. Images in Figure 17 showed the scratch wound area for both S1/TGFβ-Wnt (HLE) and S2/progenitor subtype (HepG2) cells.

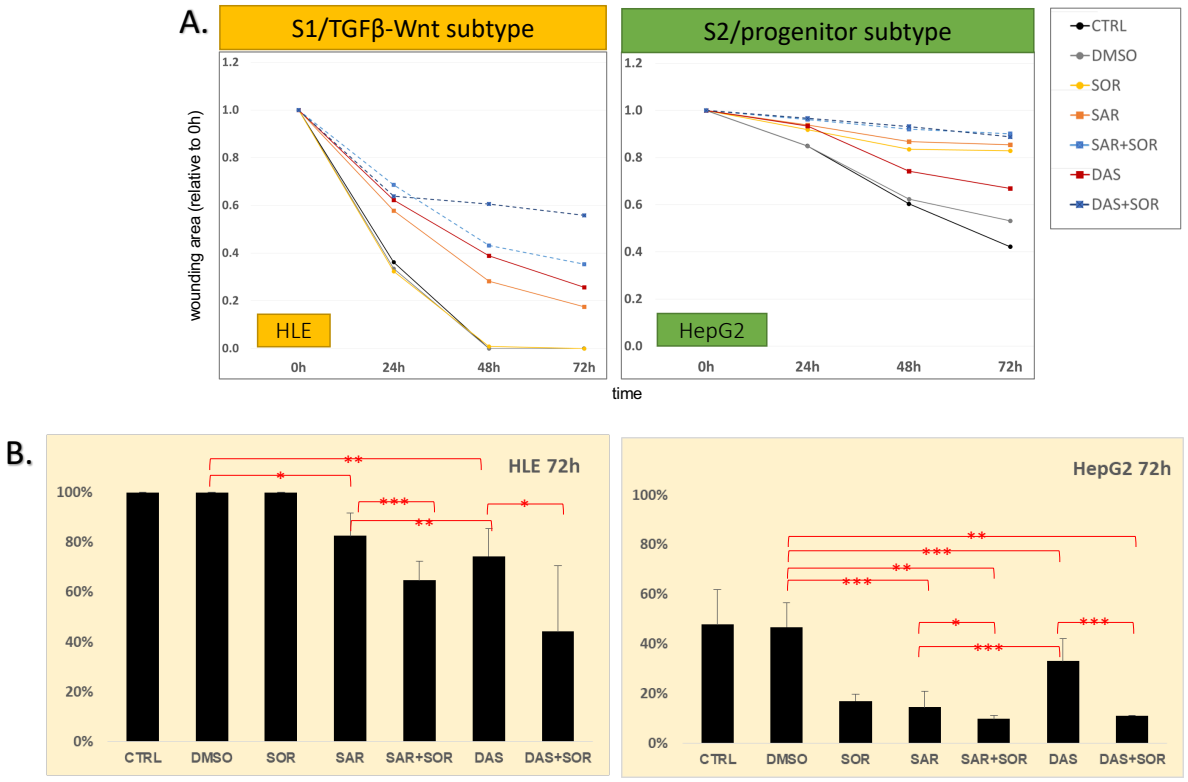
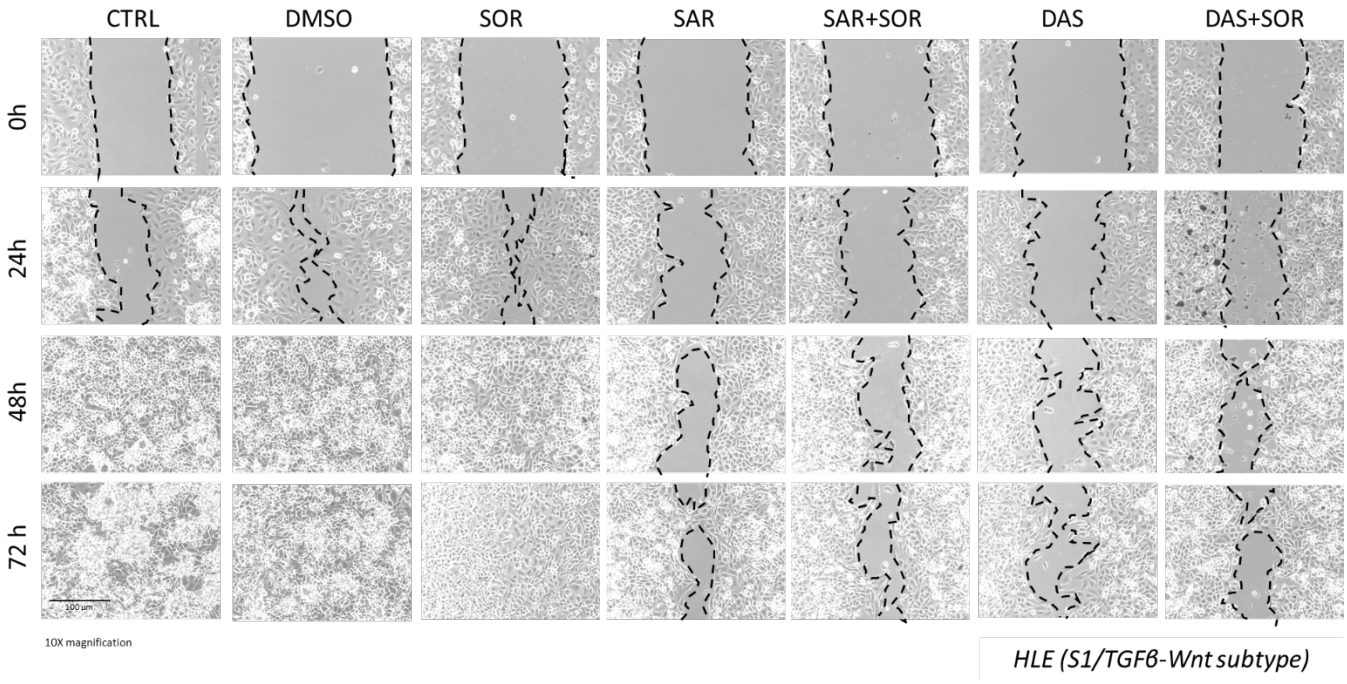


Figure 16. Quantitative analysis of wound scratch assay on the effect of sorafenib (SOR) and Src family of tyrosine kinases (SFK) inhibitors on HCC cells. (**A**) Line graphs showing wound closure area of each treatment set-up relative to 0hr measurement. (**B**) Bar graphs showing the wound closure area in % of each treatment set-up at 72 hrs after treatment. Statistical analysis for data in Figure 16B: * $p < 0.05$; ** $p < 0.01$ *** $p < 0.001$, using Student's t-test comparing treatment groups.



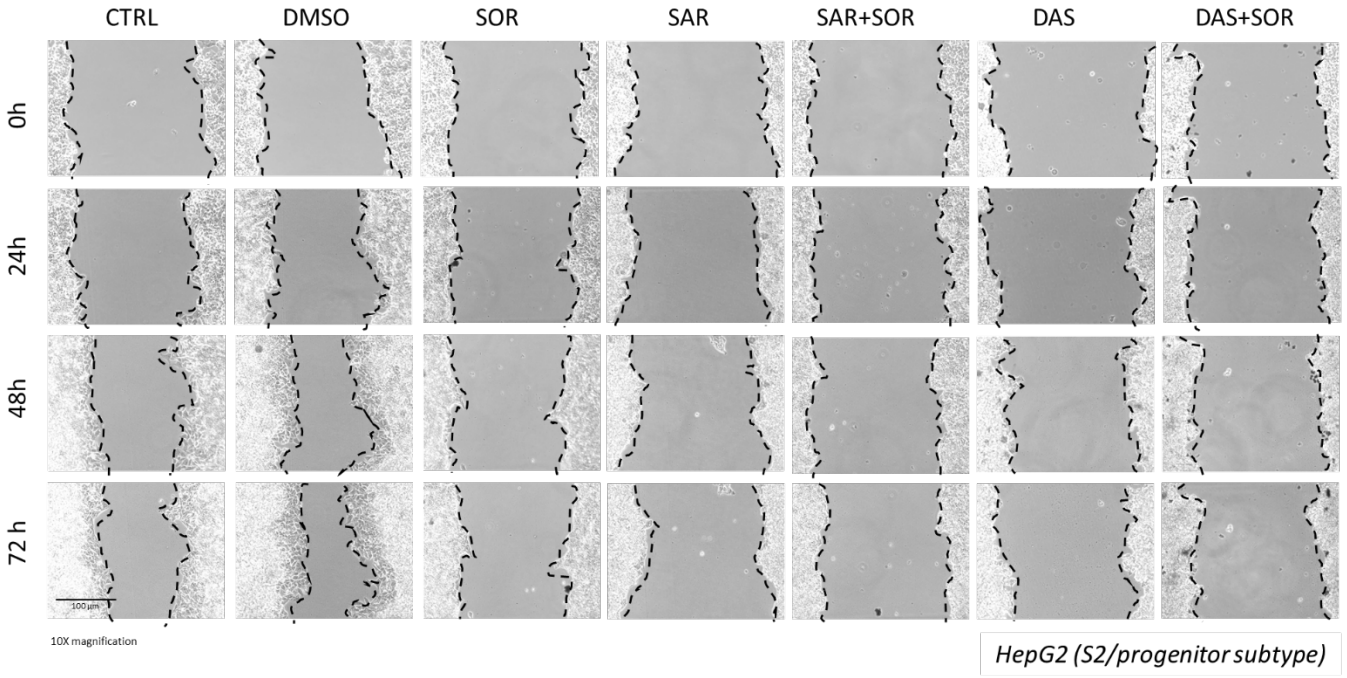


Figure 17. Wound scratch photos showing the effect of sorafenib (SOR) and Src family of tyrosine kinases (SFK) inhibitors on HCC cells. Representative images of wound scratch migration assay (0, 24 hr, 48hr, and 72 hr) showing limited wound healing in SFK inhibitors treated cells (both as mono-treatment and combined with SOR).

Chapter V - Discussion

HCC is widely known as a vast heterogeneous tumor. The innate cellular heterogeneity of HCC largely contributes to the failure of treatments. It is reported that intra-tumoral cellular and genetic differences exist from a slice of neoplastic tissue which in turn can influence the sensitivity to treatments [164]. Transcriptomic reports like the one of Hoshida *et. al* categorized HCC tumors into subclassifications. The S1 subclass shows relatively higher activation of the TGF-β pathway and cholangioma-like gene signature, and the S2 subclass is characterized by the positivity of stemness marker, EpCAM, high AFP (both serum protein and tissue gene expression levels) and GPC3, activation of IGF2 pathway. A subset of the less aggressive HCC tumors, the S3 subclass is characterized by somatic mutations accumulated in exon 3 of CTNNB1 but not with canonical WNT pathway target genes [165].

The study by Caruso *et. al* identified genetic alterations and gene expression patterns and the association with response to pharmacological agents. Using the information of molecular subclasses proposed by several groups [38,39,166], they looked into liver cancer cell lines (LCCLs) to identify markers related to drug response. In this study, the 34 LCCLs investigated reflected the molecular subclasses found in primary tumors, making them good models of heterogeneous tumors in HCC. Several inhibitors were observed to be effective against LCCLs of distinct subclass (S1, S2, and S3 subclass). Cells with overexpressed *CK19* were found to be sensitive to dasatinib. This increased expression of *CK19* is a distinct characteristic of S2/progenitor tumors. Another distinct marker of progenitor tumors is the increased expression of *IGF2*. In cells with *IGF2* overexpression, there was an increased hypersensitivity to linsitinib, an IGF1R inhibitor. Other mutational markers found in LCCLs are the activating mutations in *TSC1* or *TSC2* which suggest sensitivity to rapamycin or other mTOR inhibitors. To add, cells with a mutation in *TP53* have high sensitivity to alisertib, an Aurora kinase A (*AURKA*) inhibitor. This study was able to elaborate on specific molecular and cellular subclasses to define sensitivity to the treatment [40]. Hence it is important that in the evaluation of potential therapies and even clinical trials, these molecular subclasses are to be considered.

Several genetic changes and pathways have been reported to be altered in the hepatocarcinogenesis [167,168]. The genes most frequently mutated in HCC are *p53*, and the Wnt/wingless carcinogenesis pathways are the most frequently altered in HCC [70]. HCC is also a result of epigenetic deregulation that can be brought about by changes in DNA

methylation, histone modification, chromatin remodeling, and non-coding RNA regulation [48,169]. Using epigenetic profiling, it was possible to relate molecular subclasses to unique HCC-related epigenetic alterations. In the study of Villanueva *et al*, they observed that tumors carrying a specific signature of hypermethylated genes also harbor mRNA signatures corresponding to tumors of the S2/progenitor subclass [57]. This epigenetic signature was also found to be correlated to poor survival.

Looking at our preliminary data, *SOCS1* methylation may act as an indicator of interspatial heterogeneity in tumors. A congruent trend may not have been observed between the evaluated cohorts, but it still shows a distinct distribution of *SOCS1* methylation within the analyzed tissues. If validated, this could serve as a potential marker of the presence or absence of tissue-specific malignancy.

In our attempt to understand if the DNA methylation of *SOCS1* results in its transcriptional silencing, we also evaluated the profile of *SOCS1* mRNA expressions in the different liver tissues. We found that *SOCS1* methylation in CpG islands was not related to its mRNA expression, as it had been demonstrated previously [59]. A global integrative array study of gene expression and methylation profiling in 59 HCC patients had identified 4416 CpG sites that were differentially methylated between the tumors and their adjacent non-tumorous tissues. However, only 536 of these CpG sites were associated with differences in the expression of their associated genes [54]. As our results did not reveal any association between the gene's methylation status to its expressions. This may indicate that silencing of this TSG may be attributed to other transcriptional silencing mechanisms, other than DNA methylation. We propose the data be further verified using a computed sample size to ensure an appropriate significant investigation. The functional effect of the *SOCS1* DNA methylation should also be further evaluated to understand better the role of this aberration in the progression and aggressiveness of the disease.

Demethylation using DNMT inhibitors has been recognized as a potent epigenetic therapy. Indeed, 5-AZA was the first epigenetic drug to be approved by the FDA in the early 2000s for the treatment of myelodysplastic syndrome [170]. It has been used as a potential strategy in HepG2 cells to reverse abnormal methylation of TSGs, such as RB1, and increase gene expression activity by demethylation [171]. To add, in HCC cells LCL-PI 11 and HLE, the treatment of 5-aza-2'-deoxycytidine (5-AZA-CdR) decreased gene expressions of *DNMT1*, *DNMT3a*, and *DNMT3b* and increased *GSTP1* and *SOCS1* [172,173]. In addition, Gailhouste

et al. previously demonstrated the so-called epigenetic reconditioning using a non-cytotoxic dose of 5-AZA to induce HCC cell differentiation by increasing the expression of mature hepatocyte markers from the liver progenitor cancer cells. It reduced tumorigenicity and improved the cytotoxic effect of sorafenib [174]. It was in line with data in leukemia and other solid tumors where a low dose of 5-AZA has successfully reduced stem cell and CSC characteristics [175].

Here, we used a non-cytotoxic dose of 5-AZA to investigate the effect of demethylation in different HCC cellular subtypes and whether it would have a correlation with the expression of *SOCS1*. Since HCC is a very heterogeneous disease where DNA methylation, including for *SOCS1*, is a common event, we then evaluated whether the effect of demethylation as an epigenetic reconditioning would be effective in different cellular subtypes of the HCC [153,154,176]. In this study, we observed that a non-toxic concentration of 5 μM of 5-AZA reduced significantly the expression of DNMT1 protein in non-tumoral cells IHH and S1/TGFβ-Wnt subtypes HLE, HLF, and JHH6, but not that of S2/progenitor subtypes HepG2 and Huh7, even though DNMT1 basal expression in HCC cell lines was comparable.

Further, we showed that a non-toxic concentration of 5-AZA could restore the expression of *SOCS1*. However, its effect was dependent on the type of the cells. The *SOCS1* restoration upon 5-AZA was slightly effective only in S2/progenitor subtypes compared to S1/TGFβ-Wnt subtypes HCC, except the JHH6 cells. We did not see any significant effects of *SOCS1* expression for HLE and HLF upon 5-AZA treatment. Based on the available extensive studies on the development of targeted therapy against HCC, we predict that other agents (e.g., MET, NQO1) can be explored to reinforce the success of the epigenetic therapy [177]. Our results indicated that epigenetic reprogramming can be mostly effective in S2/progenitor HCCs that are drug-resistant with high-relapse capacity after conventional treatment. It can also be noted, as previously mentioned that S2/progenitor HCCs harbor a particular hypermethylated signature [155], therefore, epigenetic therapy can be beneficial to these molecular subclasses, and possibly lesser in S1/TGFβ-Wnt HCCs.

To compare, we also treated the cells with sorafenib, known as a dual-target inhibitor targeting the serine/threonine kinase Raf and the tyrosine kinases VEGFR/PDGFR [178]. Recently, sorafenib actions have also been associated with STAT3 regulation, where IL-6/STAT3 is involved in sorafenib-resistant hepatic CSC [179]. SOCS1 is a negative regulator of the JAK/STAT pathway, where *SOCS1* epigenetic downregulation is associated with the STAT3

activation [59,180]. A previous study showed that treatment with an anti-let-7 inhibitor increased *SOCS1* mRNA expression and increased chemosensitivity to sorafenib [181].

In this study, treatment with 50 μM of sorafenib (SOR) was able to increase *SOCS1* expression only in non-tumoral cells IHH and HCC cells JHH6. On the contrary, the *SOCS1* expression was significantly decreased in HepG2 and Huh7 cells, while, again, its expression was unchanged in HLE and HLF cells. It is important to notice that even though JHH6 is classified as an HCC cell line, it is not a tumorigenic cell line [182,183]. Furthermore, both IHH and JHH6 have low basal levels of SOCS1 compared to the other cells in this study. We hypothesize that the modulation of *SOCS1* could be influenced by its non-tumorigenic characteristics.

To summarize, we demonstrated that the DNA methylation of the TSG, *SOCS1* provides evidence of the existing interspatial tumoral heterogeneity in liver sections of HCC patients. This was demonstrated in the different distributions of DNA methylation between non-tumoral, peri-HCC, and HCC tissues. We also, explored the utility of epigenetic therapy through DNMT inhibition, as a potential treatment strategy for targeting methylation-associated targets of carcinogenesis. Epigenetic therapy using DNA methylation inhibitor 5-AZA against HCC could efficiently reduce DNMT1 protein and might restore the SOCS1. However, our results revealed that the effect of this treatment, like other treatment modalities for HCC could be cellular dependent. The prevention and reversal of *SOCS1* methylation can be a potential therapeutic target but the innate heterogeneity of HCC must still be considered.

From the result of TASK 1, we have observed that cancer treatments such as 5-AZA and SOR offer effects possibly influenced by cellular subtype. Pointing out the significance of heterogeneity in determining sensitivity to treatments. In the succeeding TASK 2, we focused our attention on identifying molecular targets that can be useful indicators to comprise the innate heterogeneity of tumors in HCC. Utilizing sets of information on HCC -omics heterogeneity [38,39], we carried out a strategy to identify potential putative markers for HCC treatment. Focusing our interest on cancer-promoting genes that are shared by the subclasses and subgroups, we evaluated, at the transcriptome level, 16 potential targets and their responses to three different treatment modalities on five different HCC cells.

Our study's data confirmed the differences between subtypes of HCC, as shown from the profile of cancer stemness markers. From baseline mRNA expression analysis of the proto-oncogene targets on the different cell lines, our results showed that the S2/progenitor subtype

displays more upregulated proto-oncogenes compared to S1/TGFβ-Wnt. This stratifies the existing differences between the two subtypes.

Upon further analysis, more prominent up-regulation in proto-oncogenes were noted, such as *SRC1*, *AURKA*, and *MDM2* in HCC cells compared to immortalized hepatocytes (Figure 2). It should be noticed that the activation, mutation, or overexpression of these genes had been reported to be involved in the hepatocarcinogenesis [184–186]. We also observed proto-oncogenes that were downregulated in the HCC cells, such as *PLZF*, *YAP1*, and *FGR*. Several publications had reported decreased expression of *PLZF* in HCC patients [131,187]. The clinical cohorts used in this study also observed the same progressive upregulation of *SRC1*, and regressive expression in *PLZF* comparing the non-tumoral, peri-tumoral, and tumoral tissues. The same trend is observed in the different stages of injury using mouse liver tissues, particularly for *SRC1*, indicating its potential as a marker of HCC progression.

We then evaluated the above targets in *in vitro* experimental models using three treatment modalities. For epigenetic therapy using 5-AZA, significant downregulations were only noticed for *FGR* in three HCC cell lines. Moreover, downregulation of *PLZF* was noticed in three cell lines, IHH, HLE, and HepG2, after 5-AZA treatment, with a significant reduction only in the HepG2 cell line. Previously, it was reported that there was no association between promoter DNA methylation and *PLZF* gene expression in liver cancer [131]. However, in contrast in pancreatic cancer, the downregulation of *PLZF* was associated with promoter DNA methylation of *PLZF* [188]. Since we showed the effect of DNA methylation inhibition on the gene expression of PLZF, our data might indicate an association between *DNMT1* and *PLZF*, at least in several HCC cell lines. However, *PLZF* regulation might be influenced by other transcriptional silencing mechanisms, not only DNA methylation. More focused studies could be explored to understand promoter methylation of target proto-oncogenes to HCC.

Regarding SOR treatment, our study showed significant proto-oncogene downregulations, mostly noticed in cells belonging to the S2/progenitor subtype HepG2 and Huh7. This could suggest that the response to SOR could be cellular/molecular subtype directed. Molecular predictors, such as *EpCAM* and tuberous sclerosis complex-2 (*TSC2*), present in S2/progenitor subtypes, dictate the response to sorafenib [189]. We had previously reviewed that cellular response to sorafenib was affected by various factors such as genetic variants and differences in dysregulated molecules in tumor cells, eventually contributing to the chemoresistance [190].

Immunotherapy is another targeted therapy that we evaluated in this study. In clinical practice, the combination between atezolizumab, an anti-*PD-L1,* and cabozantinib (anti-VEGFR) had shown potential as a first-line treatment [191]. PD-L1, expressed primarily in cancer cells, was related to HCC prognosis [192,193]. In this study, we directly targeted the *PD-L1* gene in cancer cells by silencing, which significantly reduced *PD-L1* expression. In parallel, the *PD-L1* decrease was accompanied by the downregulation of almost all investigated targets across all hepatic cells including for both HCC cell subtypes. This demonstrated an effective advantage of immune checkpoint (such as *PD-L1*) regulation compared to SOR or 5-AZA in terms of downregulating cancer-promoting genes, at least in our datasets. Our data showed that this type of immune targeting was not dependent on cellular and molecular subtypes—which can be further utilized to overcome cancer heterogeneity.

The regulation of *PD-L1* is associated with several mechanisms such as (1) alterations of genes, including *EGFR*, *ALK* fusions, *KRAS*, *MYC*, *PTEN*, and p53; (2) exogenous inflammatory cytokines, such as interferon-γ; (3) *PD-L1* amplification; and (4) disruption of the 3′-untranslated region of the *PD-L1* gene [194]. For NSCLC the overlapping mechanisms of oncogenes and immunity have been reported, for instance, changes in *EGFR* oncogenic pathway led to an upregulated *PD-L1* expression [195]. In addition, there was a positive correlation between *MET* oncogene expression and *PD-L1* expression [196], indicating a possible relationship between the two molecules. Other proto-oncogenes like *MYC* were observed to regulate the expression of two immune checkpoint proteins on the tumor cell surface – *CD47* and *PD-L1*, by directly binding to their gene promoters [197]. These are just some of the evidence of the relationship between tumor cell-associated molecules and immune dysfunction. This may suggest an overlap between oncogene-targeted therapy and immunotherapy. At present this concept still has to be fully evaluated and discussed.

In TASK 1 and TASK 2, we were able to emphasize the relevance of HCC cellular heterogeneity in response to cancer treatment. In TASK 2, the use of –omics data and bioinformatics tools, allowed us to identify relevant proto-oncogenes as useful new molecular targets of treatment. We demonstrated that immune-targeted therapy by gene silencing demonstrates a treatment advantage in overcoming cellular heterogeneity. While our data support the superiority of immune-targeted therapy vs molecular-targeted therapy and epigenetic therapy, other clinical considerations should still be considered to ensure its

efficacy. Factors like low tumor burden in HCC indicate poor response to anti-PD-L1 treatment [198].

Combination therapy involving two or more treatments to target cancer-inducing or cell-sustaining pathways is a fundamental strategy for the cancer therapy [199,200]. Monotherapy is still a common treatment modality for most cancers, however as a single-drug treatment it often has less effectiveness as compared to combination treatment. However, the issue of increased effectiveness in combination therapy may also be accompanied by increased toxicity. In contrast to monotherapy with a singular targeting approach, multiple targeting in a synergistic or additive manner allows lower therapeutic dosage for each drug [201,202]. Therefore, minimizing possible cytotoxic effects.

In TASK 3, as a continuation of our data in TASK 2, we identified the Src family of tyrosine kinases (SFKs) as a potential target for therapy. To understand the anti-tumor effects of SFK inhibitors, we evaluated saracatinib (SAR) and dasatinib (DAS) as a single treatment or combined treatment with SOR. Interestingly results of the cytotoxicity assay and wound scratch assay revealed the anti-tumor abilities of the combined treatments (SAR+SOR or DAS+SOR). The results of our experiments suggest that SFK inhibitors can enhance the activity of SOR (or *vice versa*) in targeting cell signaling pathways related to cancer growth, proliferation, and migration.

SFKs are non-receptor tyrosine kinases that bind to various proteins and mediate intracellular signal transmission. It is comprised of nine structurally similar cytoplasmic tyrosine kinases (Src, Fyn, Yes, Lyn, Blk, Fgr, Hck, Yrk, and Lck) [203]. Four members of this family of kinases were uniquely identified in our network of potential targets for HCC therapy, namely SRC1, YES, FYN, and FGR. Several reports have highlighted their roles in cancer progression and prognosis.

In HCC, SRC1 was found to promote growth and tumorigenesis of HCC cells by activating the Hippo signaling pathway [204]. The works of Feng *et al*, observed that YES activity was notably high in tumoral liver tissues of HCC patients observed with increased protein levels and enzyme activity [205]. Validation data on *FYN*, as a possible gene associated with liver cancer, showed that the expression of *FYN* was positively correlated with the prognosis of HCC patients. Data suggest that overexpression of *FYN* may act as a tumor suppressor in the presence

of malignancy, as established in malignant cells and xenograft models [206]. As for *FGR*, the report shows this marker to have prognostic value for early recurrence [207].

SFKs are involved in multiple fundamental cellular processes. The pleiotropic significance of these molecules in cellular homeostasis is associated with oncogenesis. Studies have indicated no direct association of SFKs to tumor formation [208]. However, the inhibition of SFKs activity provides an auxiliary effect of targeting primary cellular functions to eliminate cancer progression and migration [209].

Dasatinib (DAS), is the only FDA-approved Src-Abl inhibitor for use in patients with chronic myelogenous leukemia (CML) and Philadelphia chromosome-positive (Ph+) patients with acute lymphocytic leukemia (ALL) who failed first-line treatment of imatinib. Results of preclinical studies demonstrated the activity of dasatinib against solid tumors, namely in, prostate cancer [210], non-small cell lung carcinoma (NSCLC) [211], breast cancer [212], colorectal cancer [213], sarcoma [214] and melanoma [215]. The promising preclinical data on the effect of dasatinib in solid tumors prompted many phase I and phase II solid tumor clinical studies, both as monotherapy or in combination treatment [216].

Saracatinib (SAR), is another orally active, small molecule, highly selective, Src-Abl inhibitor. Same as dasatinib, it displayed promising pre-clinical anti-tumor effects in cellular models of breast cancer [217], prostate cancer [218], colorectal cancer [219], head and neck cancer [220], and lymphoma [221] solid tumors. Data of phase I clinical trials in advance solid tumors, saracatinib resulted in a maximum tolerated dose (MTD) of 175 mg daily vs 120 mg daily of dasatinib. Though both elicited tolerated dose-limiting toxicities, the ones from saracatinib even in a greater dose were easily managed [222].

Our data suggest that both DAS and SAR have better anti-tumor effects as a combination treatment with a tolerable dose of SOR in liver cancer cell lines. This was both observed in cytotoxicity experiments and migration assays. There is also a noticeable difference between the combination effects of SAR+SOR vs DAS+SOR in immortalized hepatocytes cell lines (IHH). SAR+SOR displayed a less toxic effect in IHH cells as compared to DAS+SOR, using the same combination dosage of 1.25 µM of SFK inhibitor + 10 µM of SOR. This agrees to the reported easily managed toxicity of sarcatinib in advanced solid tumors (phase I clinical trial) [222]. To our knowledge, this study presents the first pre-clinical data on the potential of combination treatment between SFK inhibitors and sorafenib in the treatment of HCC.

We have also observed that the combined effects of SFK inhibitors + SOR elicited anti-tumor effects in heterogeneous HCC cell lines to various extents. Both HLE and HepG2 appeared to be most sensitive to the combination therapy, while JHH6, Huh7, and HLF appeared to be less sensitive. While the combination treatments were effective in all, the varying extents might be influenced by molecular indicators to determine sensitivity which should be considered in further studies. This was seen also in clinical trials that were done in unselected patients. Evidence of phase II clinical trial suggested that a subpopulation of patients with advanced non-small cell NSCLC can benefit from Src inhibition. This was seen in their results suggesting *EGFR* mutations to be considered in future studies of saracatinib for NSCLC [163]. To date, many clinical trials on Src inhibitors are evaluating possible biomarkers of treatment response, to identify patients to most likely benefit from treatment [216].

Here we investigated the combined anti-tumor effect of SFK inhibitors and sorafenib in HCC malignancy. Our data demonstrate a synergistic effect of both inhibitors working on receptor tyrosine kinases (RTKs), and non-receptor tyrosine kinases (NRTKs), as sorafenib and SFKs respectively. This combined regimen resulted in enhanced control of signaling pathways involved in cell proliferation and migration (Figure 15-17). This new information could be a potential new strategy to approach HCC therapies considering heterogeneity.

Chapter VI – Conclusions and future perspectives

Despite the increase in translational studies and advances in available molecular techniques, HCC remains a malignancy of spatial global burden. This challenge could be attributed to the heterogeneous geographical incidence associated with the diverse distribution of risk factors, as well as intertumoral and intratumoral heterogeneity. In comparison to other cancers that benefited already from discoveries of biomarkers of diagnosis, prognosis, and effective targeted therapies for specific molecular aberrations, HCC still warrants translational research to improve management.

This study was able to identify epigenetic players that can be potential therapeutic targets of HCC. Epigenetic drivers of carcinogenesis can be easily identified by available tools that are cost-effective and direct (e.g. MS-PCR) and have the potential to be utilized in clinics. Epigenetic treatment can also be a new avenue to be considered in HCC therapy since exposure to a tolerable dose of DNMT inhibitor can direct a restored expression in TSGs to help reduce carcinogenesis.

Our data present evidence that sensitivity to treatment modalities can be molecular/cellular subtype driven, emphasizing the need to consider molecular classifications in the management of HCC. Most of the data generated in this study provided evidence that there is a treatment sensitivity advantage in S2/progenitor vs S1/TGFβ-Wnt activated subtype tumors (epigenetic therapy and molecularly targeted therapy). Such data adds evidence to the difference between molecular subclasses of HCC tumors, and it should be considered for identifying effective treatments. While immunotherapy can offer an effective tool to eliminate subclass-directed sensitivity, it should still be noted that not all patients are eligible for this type of treatment. Efforts should be directed at understanding how to regulate immune players and subsequently increase the possibility of patients having this type of treatment.

The use of multi-omics studies and bioinformatics tools together with experimental studies can contribute to the discovery of new targets for therapy in HCC. Although, such a strategy may be limited since published data sets tend to be saturated by the more familiar gene targets and are not able to reflect less reported or unreported targets of carcinogenesis. Moreover, the use of *in vitro* models is deemed important to reflect the heterogenous nature of HCC. Hence experimental studies should always consider different subtypes or models establishing tumor

environment and capture the true state of malignancy in tumors. As the majority of the results in this project relate to subtype-driven responses to therapies, it would be beneficial to look into the clinical translation of this subclassification of HCC tumors. Such tumor classifications might help stratify the treatment and management of HCC.

The effective monotherapy for advanced HCC is sorafenib and lenvatinib as first-line treatment. However, both drugs still offer modest overall survival benefits to patients. The recent discovery of the effective combined treatment of anti-PD-L1 and anti-VEGF provides evidence that multi-targeting of cancer-promoting mechanisms is effective for HCC management. This strategy, for now, is the best option since effective and specific biomarkers attributed to HCC development are non-existent. Hence, there is still a big gap to fill in terms of potential therapeutic targets. Enhancing the sensitivity to multi-tyrosine kinase inhibition can be a potential strategy for HCC treatment. This study was able to identify SFKs as potential targets of therapy. The combination effects of inhibitors against SFKs with sorafenib act synergistically to control cancer-promoting cellular processes. Initial evidence of a tolerable toxic effect of saracatinib and sorafenib on immortalized hepatocytes provides a good indicator of the benefit of this combination treatment. Further experiments should be considered investigating fully the mechanistic synergy between dual targeting of RTKIs (sorafenib) and NRTKIs (saracatinib) and expand this investigation in more advanced experimental models.

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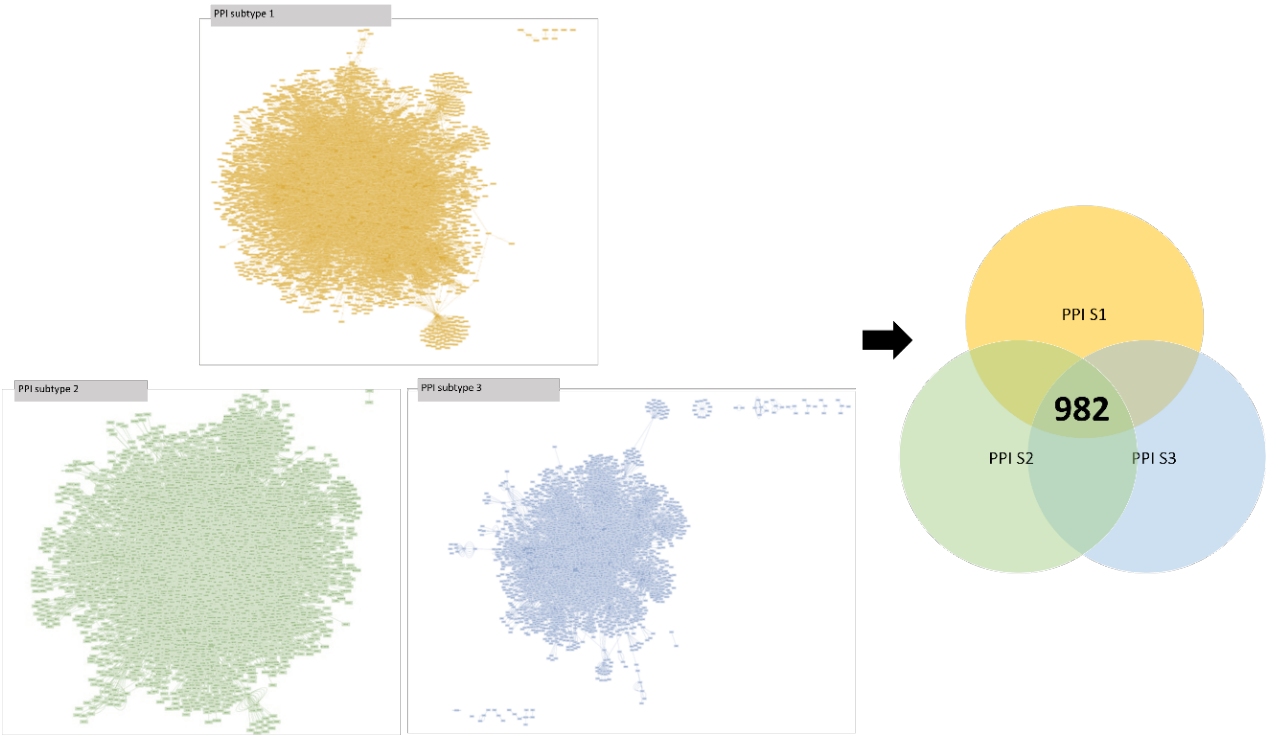
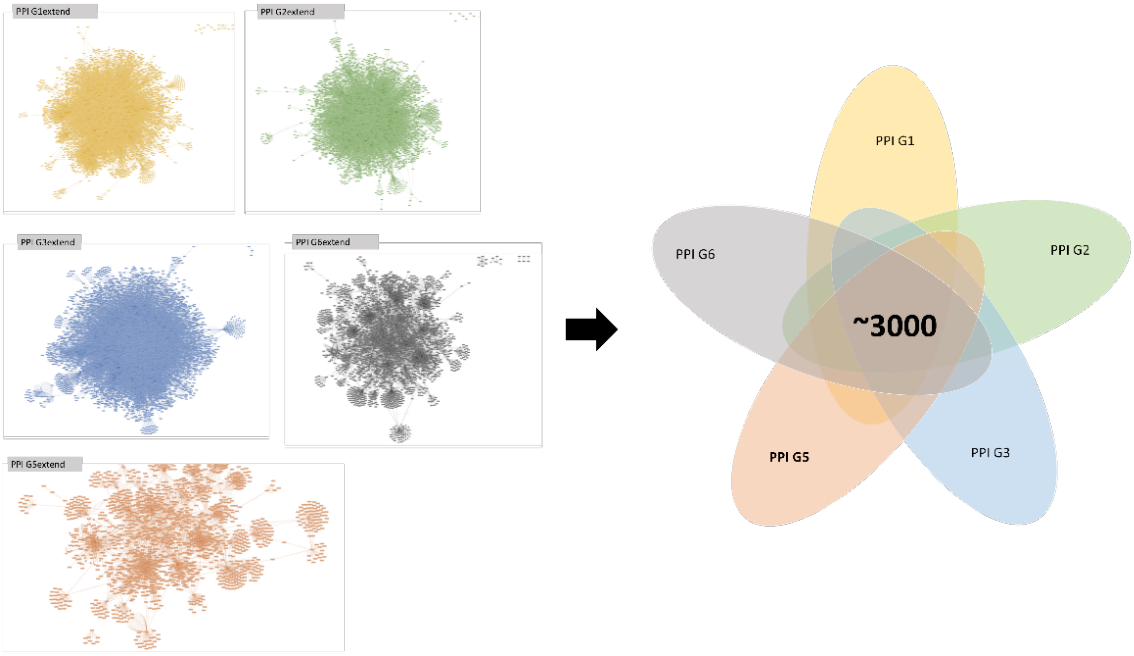
Appendix

Protein-protein interaction (PPI) analysis of the different HCC subtypes and groups from published datasets

I. Hoshida *et al.* datasets



II. Boyault *et al.* extended datasets



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I dedicate this work, to the loving memory of Nenita Dela Rosa.

I will forever miss you, Lola.

MOLECULAR PROFILING OF LIVER CANCER: GENETIC AND EPIGENETIC VARIATION ANALYSIS FOR DEVELOPING POTENTIAL TARGETS FOR THERAPY

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