Characterising the biological function of constitutive androstane receptor and its possible role in liver cancer

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STATEMENT OF ORIGINALITY

This is to certify that to the best of my knowledge; the content of this thesis is my own work.

This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Sarah Da Won Bae

Date: 21/02/2023

ACKNOWLEGDEMENT OF AUTHORSHIP

I hereby certify that the work embodied in this thesis contains a published paper(s)/scholarly work of which I am the first author.

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Chapter 3 is currently under review. I designed the study, analysed the data, and wrote the drafts of the manuscript.

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Date: 21/02/2023

AUTHORSHIP ATTRIBUTION STATEMENT

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Liang Qiao

Date: 21/02/2023

DEDICATION

I dedicate this thesis to my loved ones for their unconditional love, support, and encouragement throughout my PhD candidature.

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COVID-19 IMPACT STATEMENT

COVID-19 pandemic took place during the most crucial years of my PhD candidature with many lockdowns and restrictions, making it hard to keep my project ongoing. Despite the lockdowns and restrictions, I endeavored to finish my project and complete the tasks that can be done while I worked from home. My supervisor gave me the opportunity to write a literature review during the lockdown and this paper was recently published.

Just briefly, since the beginning of my candidature, I have been optimizing and working towards a significant sequencing experiment. Many months before the lockdown, I had already started a long-term assay to generate tumour spheres which are essential to my project. This crucial component of my project had to be ongoing for me to be able to finish my project on time. After carefully assessing the criteria for Tier 1 and Tier 2 research, I should have been eligible for permission to work on site as a Tier 1 researcher. However, despite the detailed explanation during the application process, I was not given permission and had to work from home until the end of the lockdown. I had to start all over again, waited for experimental materials to come from overseas. With the shipping and supply issues after the pandemic, it was even harder to obtain essential materials and reagents. I tried my best to catch up on lost time and fulfil all the aims of my project. Unfortunately, I did not have the time to finish the RNA-seq experiment that I had planned from the very first day of my candidature. Despite these hurdles, I was able to fulfil the rest of the aims of this project.

LIST OF PUBLICATIONS

1. **Bae, S**., George, J., & Qiao, L. (2022). From MAFLD to hepatocellular carcinoma and everything in between. Chin Med J (Engl) 135(5), 547–556.
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8. John, M., Kim, K. J., **Bae, S**., Qiao, L., & George, J. (2019). Role of BMP-9 in human liver disease. Gut, 68(11), 2097–2100.

Manuscript in submission

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CONFERENCE PRESENTATIONS

Sarah Bae, Romario Nguyen, Jacob George and Liang Qiao. Activation of constitutive androstane receptor represses cancer cell growth, migration and invasion. NSW Cancer Conference 2022, September 15-16, 2022, Sydney, Australia. [Poster] [Paper ID: 138].

Sarah Bae, Romario Nguyen, Jacob George and Liang Qiao. The constitutive androstane receptor plays A regulatory role in liver cancer stem cells. Westmead Hospital Week 2021 Research Symposium, Friday, 27th August 2021.

Sarah Da Won Bae, Romario Nguyen, Jacob George, Liang Qiao. Constitutive androstane receptor plays an important role in liver cancer via regulating liver cancer stem cells. Sydney Cancer Conference 2021, September 9-10, 2021, Sydney, Australia. [Poster and Mini-Oral] [Paper ID: 94].

Romario Nguyen, **Sarah Da Won Bae**, Jacob George, Liang Qiao. Generation and characterisation of liver specific Jagged2 knockout mice for liver cancer research. Sydney Cancer Conference 2021, September 9-10, 2021, Sydney, Australia. [Poster and Mini-Oral] [Paper ID: 95].

Romario Nguyen, Kristopher King Tao Lai, **Sarah Bae**, Jacob George and Liang Qiao. The mechanisms of acquired regorafenib resistance in hepatocellular carcinoma. Westmead Hospital Week 2021 Research Symposium, Friday, 27th August 2021.

Miya John, Kyung-Jin Kim, **Sarah Da Won Bae**, Jacob George, Liang Qiao. A Novel Molecular Differentiation Score for Hepatocellular Carcinoma Prognosis. Sydney Cancer Conference 2018, International Convention Centre, 11 - 12 October 2018, Sydney [Poster].

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SYNOPSIS

Hepatocellular carcinoma (HCC) is a global health concern and poses a great economic burden worldwide. It remains as the third-leading cause of cancer related deaths worldwide with mortality rates increasing steadily. The limited current therapeutic strategies have resulted in suboptimal responses in an alarming number of cases due to the development of drug resistance. As of now, there are no specific markers used to target HCC, therefore, an investigation into potential biomarkers is crucial to be a step closer to curative treatment. Our lab has had a special interest in identifying biomarkers for the diagnosis and treatment of liver cancer. In our preliminary work, we have identified that constitutive androstane receptor (CAR) is a possible tumour suppressor in the pathogenesis of liver cancer. In this study, we have conducted a series of complementary experiments to explore and clarify the biological roles of CAR in HCC. We have approached the hypothesis in a multi-faceted way, incorporating the use of bioinformatics and *in vitro* experiments.

An important thing to take note of when studying CAR is the species differences between the roles of CAR in animal and human settings. The published studies in animal models suggested that CAR is an oncogene in rodent liver cancer, whereas in our study, we have clearly demonstrated that CAR plays a tumour suppressor role in the pathogenesis of liver cancer by decreasing liver cancer cell proliferation, migration, invasion, and impairing the stemness of liver cancer cells. As stem cells in HCC have the ability for self-renewal, being drug-resistant, and possess a migratory phenotype, they are considered an important factor contributing to the aggressive nature of HCC. Thus, decreasing stemness features of liver cancer cells would be a key regulatory feature of CAR as a tumour suppressor.

Whilst our study shows what previous studies have not shown, that is not to say that our study is without flaws. This study clearly demonstrates the tumour suppressive role of CAR in liver cancer at a functional level, however, in-depth studies to unveil the underlying mechanism of action behind the tumour suppressive nature of CAR is essential. Future studies should investigate the underlying mechanism of action along with the clinical translatability of CITCO as a key therapeutic agent targeting liver cancer cells and stem cells.

LIST OF ABBREVIATIONS

Abbreviations	Full Term
Akt	Protein kinase B
ANOVA	Analysis of variance
BTSCs	Brain tumour stem cells
CAR	Constitutive androstane receptor
CCK-8	Cell counting kit-8
CCRP	Cytoplasmic CAR retention protein
cDNA	Complimentary DNA
CITCO	6-(4-Chlorophenyl) imidazo [2,1-b] [1,3] thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime
CO2	Carbon dioxide
CYP450	Cytochrome P450
DEN	Diethyl nitrosamine
DFS	Disease-free survival
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMA	European Medicines Agency
EPO	Erythropoietin

ERK	Extracellular-signal-regulated kinase
FDA	Food and drug administration
FGF	Fibroblast growth factor
GDC	Genomic data commons
GEO	Gene expression omnibus
GEPIA2	Gene expression profiling interactive analysis
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HNFα	Hepatocyte nuclear factor 4 alpha
HSP90	Heat shock protein 90
IPCS	International Programme on Chemical Safety
KO	Knock out
LCSCs	Liver cancer stem cells
LIHC	Liver hepatocellular carcinoma
MAFLD	Metabolic (dysfunction) associated fatty liver disease
MEK	Mitogen-activated protein kinase kinase
MeSH	Metabolic steatohepatitis
M-MLV	Moloney Murine Leukemia Virus
mRNA	Messenger RNA
MSigDB	The molecular signatures database
NR1I3	Nuclear Receptor Subfamily 1 Group I Member 3
NT	Non-tumour
OD	Optical density
OS	Overall survival

PBS	Phosphate buffered saline
PP2A	Protein phosphatase 2
PTGS2	Prostaglandin-endoperoxide synthase 2
qPCR	Quantitative polymerase chain reaction
RACK-1	Receptor for activated C kinase
RFA	Radiofrequency ablation
RGCC	Regulator of cell cycle
RNA	Ribonucleic acid
RNASeq	RNA-sequencing
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
siRNA	Small interfering RNA
T	Tumour
TBST	Tris-buffered saline and Tween20
TCGA	The cancer genome atlas
TCPOBOP	1,4-*Bis*(3,5-Dichloro-2-pyridinyloxy)benzene
TEK	TEK Receptor Tyrosine Kinase
TNFR1	Tumour necrosis factor receptor 1

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Chapter One

General Introduction

1. General Introduction

1.1 Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths world-wide (1). With an increasing trend in cases and mortality, HCC has become a significant global health-care challenge (1). Many risk factors have been identified for HCC such as hepatitis B virus (HBV), alcoholic steatohepatitis, metabolism (dysfunction) associated fatty liver disease (MAFLD), obesity and diabetes (1). Liver resection is the main curative treatment option for HCC patients and transplantation is another option for patients who do not meet the resection criteria (1). For early-stage hepatocellular carcinoma, radiofrequency ablation (RFA) is an alternative treatment (1). However, the post-treatment recurrence rate in the late-stage patient remains high (1).

Sorafenib, a tyrosine kinase inhibitor (TKI), has been approved by US Food and Drug Administration (FDA) as the first-line therapy for HCC for its inhibitory effects on Raf/serine/threonine kinases isoforms (2). However, the efficacy of Sorafenib is far from ideal and the incidence for drug resistance in patients treated with Sorafenib is high (2). Over the past few years, other drugs have been developed. For example, based on the REFLECT study, Lenvatinib has shown a better efficacy than the earlier version of TKIs in terms of median progression-free survival and overall response rate (3). Since then, Lenvatinib has been approved by the European Medicines Agency (EMA) and FDA as a first-line treatment option for patients with advanced HCC (4). Second-line therapies include regorafenib (5) and Cabozantinib (6), both have been approved by the EMA and FDA (1). Despite the recent developments in first and second-line treatment options, many patients do not respond to the available therapies. Thus, more efficacious therapeutics are needed for curative treatment of

HCC. Extensive studies are needed to unveil the mechanisms of heterogeneity of HCC and identify potential novel biomarkers for early diagnosis and therapy.

Preliminary data from our laboratory has identified the constitutive androstane receptor (CAR) as a potential biomarker of HCC. The entire thesis will focus on the role of CAR in the pathogenesis of liver cancer.

1.2. Constitutive androstane receptor (CAR) and its roles in human diseases

Constitutive androstane receptor is a nuclear receptor that is enriched in the liver with trace amounts detected in the duodenum, brain, and kidneys as shown in Figure 1 (7, 8). CAR is known to play a pivotal role in drug metabolism, energy metabolism, more recently, liver regeneration and hepatocarcinogenesis (9). The role of CAR in other diseases and pathologies have yet to be defined and still needs further exploration. Below is a summary of the role or involvement of CAR in other diseases (Table 1).

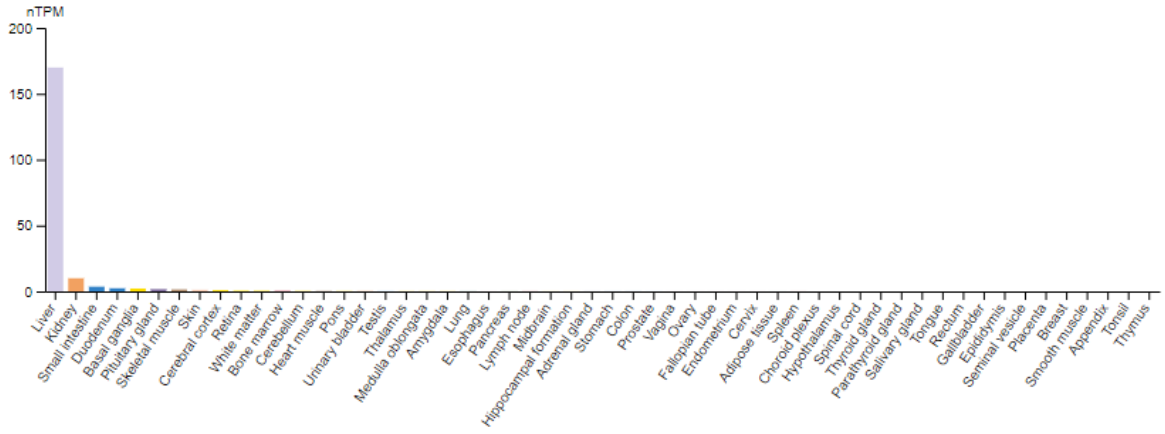

Figure 1. Tissue-wide expression of CAR. CAR is enriched in the liver with trace amounts detected in the brain, kidney, duodenum, and small intestine. nTPM, normalized transcripts per million. Image obtained from Human Protein Atlas and available from v22.proteinatlas.org/ENSG00000143257-NR1I3/tissue.

Table 1. The role of CAR in other diseases

Disease/Pathology	CAR activation can	References
Neurotoxicity	Increase mitochondrial CYP450s activity, resulting in mitochondrial dysfunction, increased oxidative stress and neurotoxicity	(10)
Lymphoma, leukemia	Induce the expression of CYP2B6. Increase drug metabolism and efficacy (e.g., cyclophosphamide)	(11)
Atherosclerosis	Prevent leukocyte recruitment into the vascular endothelium; repress initial inflammatory response; inhibit the atherogenic process	(12)
Cholesterol gallstone disease	Decrease biliary cholesterol levels, prevent cholesterol gallstone formation	(13)
Inflammatory bowel diseases	Accelerate intestinal epithelial wound healing in cell lines; promote mucosal healing in experimental colitis; Inhibit proinflammatory signaling	(14)
Brain tumour	Repress brain tumour stem cell growth and expansion. Play a tumour suppressor role in brain cancer	(15)

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Review

Role of the constitutive androstane receptor (CAR) in human liver cancer

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ABSTRACT

Background: The constitutive androstane receptor (CAR) is a member of the nuclear receptor superfamily (subfamily 1, group I, member 3, also known as NR1I3) that is almost exclusively expressed in the liver. CAR interacts with key signalling pathways such as those involved in drug, energy and bilirubin metabolism. In mouse models, activation of CAR leads to tumorigenesis by inducing pro-proliferative and anti-apoptotic signalling. However, many previous reports have shown species differences between CAR activity in animal models and humans. Recent studies have demonstrated that the mode of action of CAR in rodent liver tumorigenesis is not applicable to humans. Despite this, many studies still continue to study the role of CAR in animal models, hence, there is a need to further explore the role of CAR in human diseases particularly cancers. While there is limited evidence for a role of CAR in human cancers, some studies have proposed a tumour-suppressive role of CAR in liver cancer. In addition, recent studies exploring CAR in human livers demonstrated a hepato-protective role for CAR in and more specifically, its ability to drive differentiation and liver regeneration. This review will discuss the role of CAR in liver cancer, with a focus on species differences and its emerging, tumour-suppressive role in liver cancer and its role in the regulation of liver cancer stem cells.

1. Introduction

The constitutive androstane receptor (CAR) is a member of the nuclear receptor superfamily (subfamily 1, group I, member 3, also known as NR1I3) [1] and is enriched in liver [2]. RNA expression summary from the Human Protein Atlas (http://www.proteinatlas.org) shows that NR1I3 is predominantly expressed in liver with trace amounts detected in other organs such as duodenum, brain, heart and kidneys [2]. CAR was discovered as an xenosensor [3] of both endogenous and exogenous ligands. Following its discovery, studies have identified downstream targets and roles for CAR in drug [4–15] and energy metabolism [16–21] (Table 1) (as well as tumorigenesis in rodents). Previous studies have shown that CAR is activated by two pathways: ligand-dependent ("direct") and ligand-independent ("indirect") [22]. Unlike the other members of the nuclear receptor superfamily, CAR possesses dual functions with key roles in drug metabolism and xenobiotic clearance. The discovery of its structure and ligands allowed exploration of the role of CAR in human diseases, particularly in cancers [23]. Members of the cytochrome P450 (CYP450) family and genes that control cellular proliferation and apoptosis are the main downstream targets of CAR (Table 1) and are relevant to liver tumorigenesis in mice [23]. Animal models have demonstrated that activation of CAR by its indirect activator phenobarbital and ligand TCPOBOP (1,4-Bis-[2-(3,5-dichloropyridyloxy)] benzene, 3,3′,5,5′-Tetrachloro-1,4-bis (pyridyloxy) benzene), with subsequent nuclear translocation, induces liver cancers in mice [23].

Recently, epidemiological and comparative studies have shown clear species differences between human and rodent CAR in liver cancer. These collectively indicate that the rodent mode of action in liver tumorigenesis is not applicable to humans [23,24]. Despite the lack of relevance to humans, many studies still report the role of CAR in rodent liver cancer [25], including in metabolic (dysfunction) associated fatty liver disease (MAFLD) related liver cancer [26], while very few studies have explored CAR in human liver cancer. Recently, a more interesting role for CAR in protecting the liver during development and regeneration has surfaced but this needs to be further explored [27]. Another study on brain tumour stem cells (BTSCs) has demonstrated a tumour-suppressive role of CAR in humans [28]. This review will discuss species differences and the emerging tumour-suppressive and protective roles of CAR to highlight the need for more research exploring its role in human liver cancer.

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Table 1
Downstream target genes for mouse and human CAR in drug and energy metabolism.

Class	Mouse	Human
Phase 1 drug-metabolizing enzymes	Cyp1a1, Cyp1a2, Cyp2a4, Cyp2b10, Cyp2c29, Cyp2c37, Cyp2c55, Cyp3a11, Nqo1, Aldh1a1, Aldh1a7, Akr1b7, Ces6	CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP3A4, CYP3A5
Phase 2 drug-metabolizing enzymes	Ugt1a1, Ugt1a9, Ugt2b34, Ugt2b35, Ugt2b36, Sult1e1, Sult2a1, Sult2a2, Sult3a1, Sult5a1, Gsta1, Gsta4, Gstm1, Gstm2, Gstm3, Gstm4, Gstp, Gstt1	UGT1A1, SULT2A1
Drug transporters	Mrp2, Mrp3, Mrp4, Oatp1a4	MDR1
Energy Metabolism	Insig-1, SREBP1, HNF4A, FOXO1, PEPCK, G6Pase	

2. Activation of CAR

2.1. Ligand-dependent (or direct) activation of CAR

Ligand-dependent activation involves the binding of known ligands to CAR (shown in Table 2) [29–38]. Upon binding to CAR in the cytoplasm, the ligand triggers recruitment of protein phosphatase 2A (PP2A), causing dephosphorylation and subsequent dissociation of CAR from its chaperone proteins such as heat shock protein 90 (HSP90) and cytoplasmic CAR retention protein (CCRP), leading to nuclear translocation of free CAR. The translocated CAR heterodimerizes with the retinoid X receptor (RXR) in the nucleus [22].

2.2. Ligand-independent (or indirect) activation of CAR

In this pathway, ligand binding is not essential for activation. Here, CAR activation associates with epidermal growth factor receptor (EGFR) signalling [39]. In the absence of a ligand, epidermal growth factor (EGF) binds to EGFR, triggering a signalling cascade where mitogen-activated protein kinase kinase (MEK) and src-kinase are both activated. MEK activates extracellular-signal-regulated kinase (ERK) which prevents nuclear translocation of CAR and activated src-kinase inhibits receptor for activated C kinase (RACK-1) signalling. In the presence of a ligand, the CAR activators compete with EGF in binding to EGFR on the cell surface and antagonizes subsequent activation of its downstream pathways. For example, the CAR ligand phenobarbital binds to EGFR and favours CAR signalling pathway by negatively regulating MEK/ERK and src-kinase activity. Subsequently, inhibition of src-kinase negatively regulates RACK-1 which allows for PP2A recruitment for nuclear translocation of CAR.

Table 2
Identified ligands and activators for CAR.

Chemicals	Species	Source	Activity	References
CITCO	Human	Synthetic	Agonist	[29]
Phenobarbital	Human	Synthetic	Indirect activator	[30]
Androstanol	Human	Synthetic	Inverse agonist	[31]
CINPA-1	Human	Synthetic	Antagonist	[32]
Valproic acid	Human	Synthetic	Agonist	[33]
Efavirenz	Human	Synthetic	Agonist	[34]
Flavonoids	Human	Natural polyphenols	Agonist	[35]
TCPOBOP	Murine	Synthetic	Agonist	[36]
Androstanol	Murine	Synthetic	Inverse agonist	[31]
Phenobarbital	Murine	Synthetic	Indirect activator	[30]
Paclitaxel	Murine	Natural	Agonist	[37]
Diallyl sulfide	Murine	Natural	Agonist	[38]

Abbreviations: CINPA-1, Ethyl [5-[(diethylamino)acetyl]-10,11-dihydro-5*H*-dibenz[*b*,*f*]azepin-3-yl]carbamate; CITCO, 6-(4-Chlorophenyl) imidazo [2,1-b] [1,3] thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; TCPOBOP: 1,4-Bis-[2-(3,5-dichloropyridyloxy)] benzene, 3,3′,5,5′-Tetrachloro-1,4-bis (pyridyloxy) benzene.

2.3. Known ligands and activators for mouse and human CAR

To date, numerous exogenous and endogenous CAR ligands and an indirect activator have been reported in humans and rodents (Table 2). They range from synthetic drugs to natural compounds, but it is important to note that some ligands may exert cross-species activity when used in high doses, meaning that they can activate CAR-responsive genes in both rodents and humans [40] (Table 2). For example, TCPOBOP is a well-known murine-specific CAR activator, but at high doses it can activate downstream targets in rat liver [40]. CITCO (6-(4-Chlorophenyl) imidazo [2,1-b] [1,3] thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime), a human-specific CAR activator has also been shown to induce CAR downstream genes in murine liver [41].

3. The role of CAR in rodent HCC

The role of CAR signalling in hepatocarcinogenesis is controversial. Treatment of wild-type mice with the CAR activators phenobarbital or TCPOBOP, leads to CAR activation together with enhanced DNA replication and suppressed apoptosis [42–44]. This is likely mediated by activation of Gadd45β signalling [44–46]. Indeed, phenobarbital-induced CAR activation in wild-type mice leads to hepatocarcinogenesis through similar mechanisms including activation of Gadd45β, induction of CYP2B, increased cell proliferation, hepatic hypertrophy and enhanced anti-apoptotic signalling [45]. Further studies have verified that CAR activation but not the activation of tumour necrosis factor receptor 1 (TNFR1) and NF-κB signalling pathways is responsible for Gadd45β upregulation in response to TCPOBOP [46]. Apart from Gadd45β, activation of CAR by TCPOBOP in rats leads to activation of c-Myc, a critical transcription factor involved in the promotion of cell proliferation, angiogenesis, and inhibition of apoptosis [47]. Several other types of CAR activators, for example, gingko biloba extract [48], triazole fungicides [49], synthetic pyrethroid momfluorothrin [50] and toxaphene [51] can promote tumour formation especially hepatocarcinogenesis. Collectively, CAR is a tumour promoter in rodents where activation leads to signalling that promotes uncontrolled cell proliferation and suppresses apoptosis.

It has been shown previously that a significant amount of phenobarbital-treated DEN induced tumours in mice harboured activating mutations of β-catenin [52]. Similar effects were observed in TCPOBOP treated DEN-induced liver cancer in mice [53]. Ctnnb1 (a gene encoding for β-catenin) knock-out in phenobarbital treated, DEN-induced tumours in male mice did not show any increase in CAR dependent cell proliferation [54]. The role of CAR and β-catenin dependent signalling driving tumour cell proliferation and growth has been explored further. Long-term activation of CAR led to an increase in β-catenin activity and uncontrolled cell proliferation [55]. Furthermore, gene expression of NR1I3 decreased in the absence of hepatic β-catenin and the opposite effects were seen when β-catenin expression was slightly increased [55]. There seems to be an interaction between CAR and β-catenin that drives uncontrolled hepatocyte expansion, ultimately leading to tumour growth in mice, however, these results were not observed in primary human hepatocytes or human liver cancer cell lines [55]. It is now well established that CAR activation leads to hyperplasia and more recently was linked to β-catenin and the protein kinase B (Akt) pathway [21]. This study also showed significant functional synergism between CAR and β-catenin whereby prolonged activation of CAR in mice leads to increased β-catenin levels and its transcriptional activity along with hyperplasia [21]. CAR and β-catenin share the same role as the drivers of uncontrolled proliferation and tumour growth in mice and work synergistically in doing so. However, the exact mechanism of

action behind this interaction is unknown [56]. Collectively, CAR is a tumour promoter in rodents where activation and/or interaction with other key players promotes uncontrolled cell proliferation, tumour growth, cancer cell survival via suppressing apoptosis.

4. The role of CAR in human HCC

The role of CAR in human liver cancer has not been extensively studied. Based on limited reports, human CAR appears to exert different or even opposite effects compared to its rodent counterpart (Fig. 1). For example, activation of CAR by phenobarbital in human hepatocytes does not alter cell proliferation in vitro, neither does it affect the behaviour of hepatocytes in chimeric mice with humanized liver in vivo [57–59]. This functional species discrepancy raises the question as to whether the mechanism of action of CAR in rodent HCC development is directly applicable to humans.

The species differences in the susceptibility of liver cells to CAR activation and subsequent liver cancer formation was explored in wild-type, humanized mouse models and cultured mouse and human hepatocytes following phenobarbital treatment [60]. In wild-type and humanized mouse hepatocytes, when murine CAR is replaced with its human counterpart, activation of CAR by phenobarbital led to increased liver weight, liver hypertrophy, CYP450 activation and enhanced DNA replication [60]. However, when human primary hepatocytes were treated with phenobarbital to activate CAR, only an increase in CYP450 expression was observed. Similarly, activation of human CAR on a murine background by phenobarbital only led to hypertrophy. In contrast, in wild-type mice, activation of CAR by phenobarbital not only led to liver hypertrophy, but also to hepatocyte hyperplasia and activation of cell proliferation related genes [61]. More recently, by using cross-species comparisons in mouse and human 3D liver microtissues, activation of CAR by phenobarbital did not result in pro-carcinogenic events in human liver [62].

The International Programme on Chemical Safety (IPCS) published a framework to better analyse the relevance of animal models of action in carcinogenesis for humans [63]. A previous study [64] examined the existing mode of action of phenobarbital-induced rodent liver tumorigenesis using the IPCS framework [57,61]. Many other studies, more specifically epidemiologic studies, have attempted to examine the relevance of phenobarbital-induced rodent liver tumorigenesis to humans. These studies have failed to reveal a significant association between prolonged phenobarbital exposure in patients and an increased risk of liver cancer [24,65–67]. Overall, activation of human CAR does not seem to have the same mitogenic effects as in rodents and it is likely that the mechanisms of action mediated by CAR activation is murine specific [25].

In a genome-wide study, changes in gene expression patterns in HepaRG-KO CAR cells treated with phenobarbital or CITCO were compared [68]. It was found that both agents suppressed the expression of growth promoting genes such as TEK (TEK tyrosine kinase, endothelial), RGCC (regulator of cell cycle) and PTGS2 (prostaglandin-endoperoxide synthase 2). Thus, CAR activation suppresses cell growth-promoting genes and may play a role in negatively regulating cancer cell or cancer stem cell growth in humans.

5. CAR and liver cancer stem cells (LCSCs)

Cancer stem cells are a small subset of cancer cells with the characteristics of normal stem cells. They have the ability of long-term self-renewal and are highly tumorigenic [69–71]. Cancer stem cells

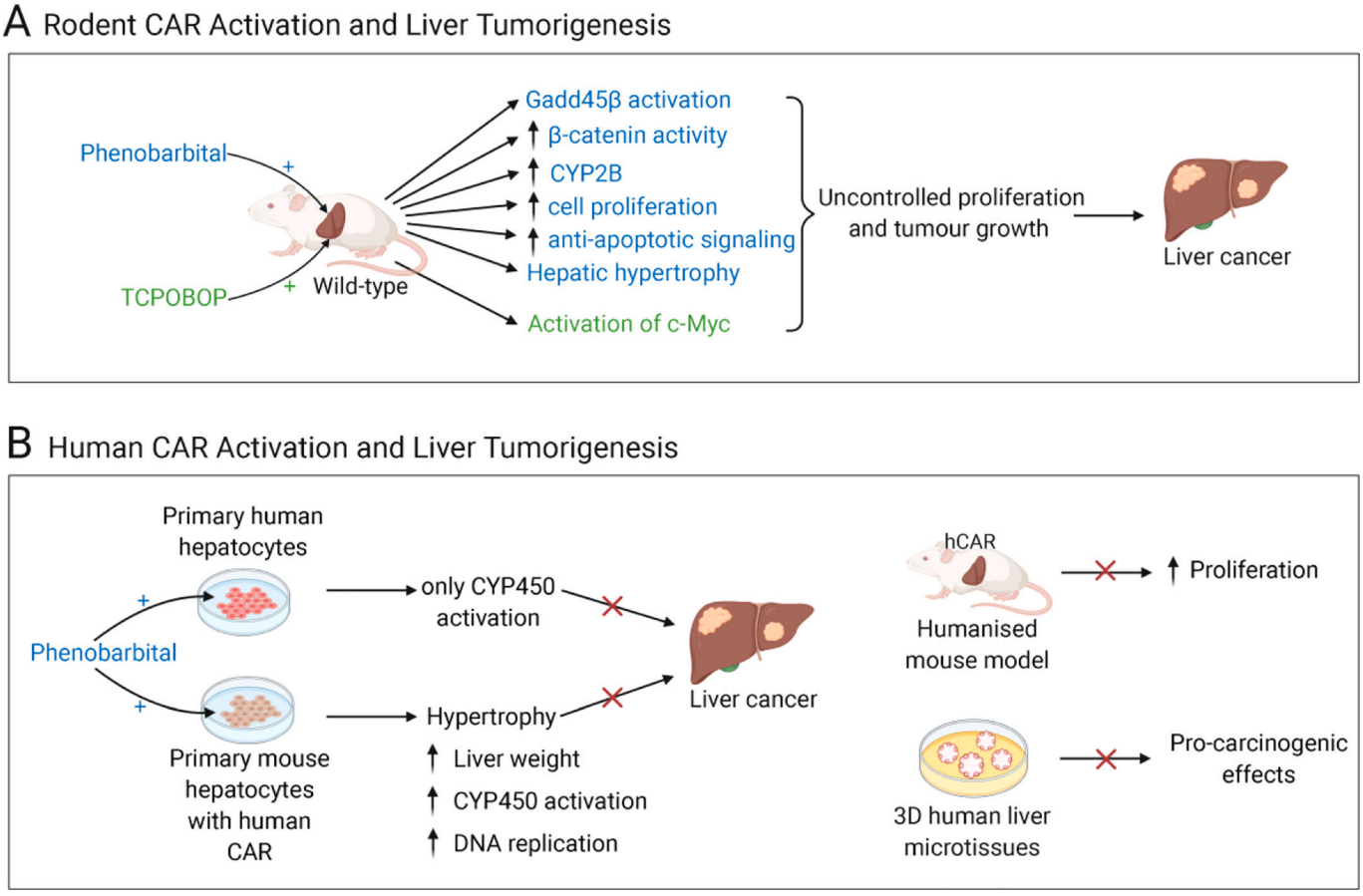

Fig. 1. A diagram comparing the events following CAR activation in rodent and human liver cancer. A. Activation of rodent CAR by TCPOBOP and phenobarbital in rodents leads to liver tumorigenesis. B. Activation of human CAR by phenobarbital does not induce liver cancer. Diagram was created with BioRender.com.

contribute greatly to cancer initiation, metastasis, drug-resistance and relapse. Multiple signalling pathways are involved in the maintenance of the stemness features of CSCs, such as Notch, Wnt and Hedgehog pathways [72]. A previous study highlighted that nuclear receptors similar to CAR, were involved in the regulation of stemness and adult stem cells in general [73], but no studies have investigated the role of CAR in liver CSCs (LCSCs).

Under physiological conditions, stem and progenitor cells differentiate into liver specific cells [74]. Any faults in this regeneration process including genetic and epigenetic alterations, can result in de-differentiation of hepatic cells into LCSCs [74]. Interestingly, it has been reported that hepatic differentiation is CAR-specific where CAR expression is increased during differentiation along the hepatic lineage and enhanced CAR expression promotes hepatic differentiation [27]. It was also proposed that CAR is a promotor of stem cell differentiation and maturation to hepatic-like cells [27]. The process of liver injury-regeneration is complex and multi-faceted and any mishaps during this process can result in the formation and mobilization of LCSCs [74]. Studies investigating the effects of CAR activation on liver regeneration have reported that treatment of ex-vivo cultures of injured liver tissue slices with CITCO led to improved histological appearance, enhanced proliferative responses and greater cell viability [75,76], demonstrating a protective role of CAR in the liver regeneration process. As of now, there is limited evidence for a definite role of CAR in LCSCs.

However, a study in human brain tumour has revealed that activation of CAR signalling in human BTSCs by CITCO (a CAR agonist) exerted a significant inhibitory effect on the growth and expansion of the BTSCs and the differentiated brain tumour cells [28]. Mechanistically, CAR activation by CITCO resulted in cell cycle arrest and apoptosis in BTSCs only, and a dose-dependent decrease in the proportion of CD133+ BTSCs and a significant reduction in CD133 expression in BTSCs [28]. The results of this study collectively suggest that CAR may act as a tumour suppressor, through the inhibition of BTSC growth and expansion.

Based on the above available data, we propose that CAR may play a possible or tumour-suppressive role in human liver cancer. Since CAR plays an important role in driving differentiation along the hepatic-lineage [27] as well as driving liver regeneration post injury [75,76], it may have an antagonistic role in LCSC formation by aiding the differentiation of stem and progenitor cells and/or hindering the de-differentiation of the differentiated hepatic cells into LCSCs. It is also possible that CAR activation could inhibit the expression of key CSC markers such as CD133 and affect its downstream cascades such as LCSC expansion. More detailed studies need to be done to investigate the role of CAR in LCSCs.

6. Conclusions

The oncogenic role of CAR in rodent liver cancer is well reported. However, its role in human liver cancer has not yet been defined. Many comparative and epidemiological studies show that mode of action for CAR activation-induced hepatocarcinogenesis in rodents is not applicable to humans. Therefore, there is a need to further explore the role of CAR activation in human liver cancer and more specifically, on LCSCs. Previous studies have demonstrated that CAR drives the differentiation of stem and progenitor cells, protects liver injury, and promotes liver regeneration. However, the underlying mechanisms are poorly defined. As CAR is almost exclusively expressed in the liver, and it interacts with multiple other key signalling pathways, we speculate that CAR may function as a tumour suppressor by suppressing LCSC activity or hindering de-differentiation of differentiated cells into LCSCs, as well as inhibiting the key markers for LCSCs such as CD133. A better understanding of the role of CAR in LCSCs and liver cancer will allow us to discover the specific role of CAR in human hepatocarcinogenesis. Hence, more studies are clearly warranted.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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1.3 Role of CAR in benign and pre-cancerous liver diseases

Metabolic (dysfunction) associated fatty liver disease (MAFLD) is the hepatic manifestation of systemic metabolic dysregulation and is an umbrella term encompassing a range of liver disease states from hepatic steatosis to metabolic steatohepatitis (MeSH) (16). Drug metabolism regulates hepatic lipid biosynthesis directly by inducing insig-1 which regulates cholesterol metabolism, lipogenesis and glucose homeostasis (17). Amongst obese steatosis and MeSH patients, drug metabolism is impaired as the accumulation of liver lipids significantly downregulates the expression and activity of Cytochrome P450s (CYP450) (18). In diabetes, CYP450s are heavily involved in the biotransformation of drugs and cholesterol (17). Diabetic patients undergone chronic phenobarbital, a CAR agonist, treatment had lower plasma glucose levels coupled with better insulin sensitivity (19, 20). In a more recent study, activation of human CAR in human hepatocytes by two novel activators (UM104 and UM145) selectively inhibited gluconeogenesis without suppressing fatty acid synthesis (21). They also found that activation of CAR decreased production of glucose in the liver (21). Interestingly, this study also found species discrepancies between CAR induced changes to energy metabolism in human and mouse primary hepatocytes which further highlights the conclusion that mode of action of liver tumorigenesis as defined in animal models, cannot be applied to humans. Previous studies have investigated the role of CAR in liver diseases and as CAR is a well-known xenosensor, we can suggest that the activation of CAR will lead to an increase in drug metabolizing enzymes to attenuate or improve liver diseases.

1.4 Summary and conclusion

CAR plays a key regulatory role in the liver being responsible for drug and energy metabolism, as well as plays a hepatoprotective role during liver injury and would healing process. However, the role of CAR in human cancers has been very scarcely studied. In the context of liver cancer, CAR has been found to play an oncogenic role in rodent liver but in humans, CAR

appears to play a tumour suppressor role. CAR was shown to act as a tumour suppressor in human brain cancer. However, the definite role of CAR in human liver cancer remains inconclusive. Hence, extensive studies are needed to better understand the functional role of CAR in human cancers.

1.5 Aims and Hypothesis

In this project, we aim to investigate the role of CAR in human liver cancer and clarify its mechanism of action using a wide range of methods to modulate CAR to be used for various functional assays. We have employed CAR knockdown and overexpression models of human HCC cell lines, Hep3B, Huh-7 and PLC/PRF/5. We will also be using a CAR agonist to activate human CAR in human HCC cell lines mentioned above. Functional assays such as proliferation, cell cycle, migration, invasion and tumour sphere formation assays have been used to study the role of CAR in liver cancer at a functional level.

We hypothesize that CAR plays a tumour suppressor role in human liver cancer, and this is likely through regulating the functional aspects of cancer growth and progression with a special focus on liver cancer stem cells (LCSCs).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Bioinformatic Analysis

2.1.1. Microarray analysis of CAR expression and biological implications in normal and tumour tissues.

RNASeq data for nuclear Receptor Subfamily 1 Group I Member 3 (NR1I3) mRNA expression in human normal liver and HCC tissues were downloaded from the following GEO datasets: GSE14520, GSE22058, GSE25097, GSE36376, GSE57957, GSE57958, GSE60502. GEO dataset GSE89377 was used to determine gene expression levels in various HCC stages and grades. Sample numbers and platforms for each GEO dataset used are listed in Table 2. Microarray data for NR1I3 gene expression in human normal liver and HCC tissues, and other clinical information were downloaded from GDC TCGA LIHC (n=412) (MEXPRESS: https://mexpress.be/) and LIHC TCGA Firehose Legacy (n=442) (cBioPortal: https://www.cbioportal.org/). Prior to downloading data from MEXPRESS, filters were applied to omit samples with null expression of NR1I3 and to only include cases of primary liver cancer.

2.1.2. Survival analysis

Data for patient overall survival (OS) and disease-free survival (DFS) and their respective hepatic NR1I3 mRNA expression levels were downloaded from GDC TCGA (OS only), TCGA, Firehose Legacy and GSE14520. Samples were arbitrarily split into two groups: low and high NR1I3, using Cutoff Finder, an online tool to determine cut-off points in molecular data (https://molpathoheidelberg.shinyapps.io/CutoffFinder_v1/). Survival rates were compared between $NR1I3^{low}$ and $NR1I3^{high}$ samples using the Kaplan-Meier method and log-rank test.

2.1.3. Correlation between NR1I3 and gene sets obtained from MsigDB and cBioPortal

Gene sets related to cancer prognosis, survival, recurrence, progression, proliferation, invasion, metastasis, angiogenesis, tumour immune response, stem cell features, signalling pathways involved in cancer growth and survival, as well as HCC specific gene sets were downloaded from Molecular Signatures Database V7.0 (MSigDB) and cBioPortal. Gene signatures that were obtained from cBioPortal have been given the prefix "cBioPortal" and those obtained from MsigDB were assigned the studies' author as the prefix. The top 20 genes associated with favourable and unfavourable survival, total of 2 gene sets, for liver cancer were obtained from Protein Atlas and were given the prefix "Protein Atlas". Spearman correlation between NR1I3 expression and gene sets for LIHC were analysed using Gene Expression Profiling Interactive Analysis 2 (GEPIA2). Negatively and positively correlated gene sets were separated into two groups and within each group, the gene sets that met the inclusion criteria were selected. In this study, Spearman's correlation of -0.5 and 0.5 represents a moderate negative and positive correlation, respectively. Any gene sets that did not have a moderate correlation to CAR were omitted. A p value of ≤ 0.05 is considered statistically significant.

Table 2. Platforms and sample numbers used in this study

GEO Accession	Platform	NT	T
GSE14520	GPL3921 (Affymetrix HT Human Genome U133A Array)	220	225
GSE22058	GPL6793 (Rosetta/Merck Human RSTA Custom Affymetrix 1.0 microarray)	97	97
GSE25097	GPL10687 (Rosetta/Merck Human RSTA Affymetrix 1.0 microarray, Custom CDF)	243	243
GSE36376	GPL10558 (Illumina HumanHT-12 V4.0 expression beadchip)	193	240
GSE57957	GPL10558 (Illumina HumanHT-12 V4.0 expression beadchip)	39	39
GSE57958	GPL8490 (Illumina Human Methylation 27 beadchip (HumanMethylation27_270596_v.1.2)	59	61
GSE60502	GPL96 ([HG-U133A] Affymetrix Human Genome U133A Array)	18	18

GEO (gene expression omnibus), HCC (hepatocellular carcinoma)

NT: non-tumours; T: tumours

2.2 Cell Culture

Three human HCC cell lines including Hep3B, Huh-7 and PLC/PRF/5 were obtained from Cell Bank Australia. Hep3B and PLC/PRF/5 were cultured in Dulbecco's Modified Eagle Medium (DMEM; Lonza, Basel, Switzerland) supplemented with 10% FBS in a 5% CO_2 incubator at 37°C. Huh-7 was cultured with DMEM with low glucose (Sigma-Aldrich; St Louis, MO, USA, D6046) supplemented with 10% fetal bovine serum (FBS; Gibco, Waltham, Massachusetts, United States) in a 5% CO_2 incubator at 37°C. Cells have been tested for mycoplasma (negative) and STR profiling was done.

2.3 Activation and modulation of CAR expression in HCC cell lines

2.3.1 Activation of CAR

Cells were treated with CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime, a CAR agonist; TOCRIS, Bristol UK). Briefly, cells were exposed to 1 μM of CITCO in growth media for the indicated time and DMSO was used as vehicle control. Effect of CITCO on CAR activation was confirmed by quantifying the gene and protein expression of CAR specific downstream targets including CYP2A6, CYP3A4 and UGT1A1.

2.3.2 Modulation of CAR expression

Expression of CAR in HCC cell lines were down-regulated by using CAR specific siRNAs or overexpressed by using a plasmid carrying human CAR encoding gene NR1I3. The optimal concentration of CAR specific siRNAs (Dharmacon, Lafayette, CO, USA) to be used in three HCC cell lines was pre-determined. Based on our titrations, 40 nM of siRNA was found to be sufficient to induce >60% knockdown on CAR expression and thus was used in the subsequent studies. For subsequent knockdown studies, cells were seeded onto 6-well plates and grown till

70% confluency. Cells were then transfected with CAR specific siRNA for 48 h before functional assays as described below.

To overexpress CAR, cells were transfected with the expression plasmid containing human CAR encoding gene NR1I3 (pCMV6-Entry-NR1I3) (Origene Technologies, Rockville, Maryland, US) in Opti-MEM reduced-serum containing FuGene (Promega, Wisconsin, US). Cells were transfected for 48 h prior to functional assays. Successful knockdown and overexpression of CAR was confirmed by qPCR and western blots.

2.4 RNA extraction, cDNA synthesis and quantitative real time RT-PCR (qPCR)

Total RNA was extracted using FavorPrep Tissue Total RNA Kit (Favorgen, Taiwan) according to the manufacturer's protocol. Extracted RNA was used to generate cDNA using M-MLV reverse transcriptase (Promega, Wisconsin, US). cDNA was diluted (1:10) and used for qPCR with QuantiNova SYBR Green PCR Kit (Qiagen, Hilden, Germany). qPCR cycling conditions included initial activation step (2 min, 95^{o}C), denaturation (5s, 95^{o}C) and combined annealing/extension (10s, 60^{o}C) for 35-40 cycles. qPCR was performed using the CFX96 Touch Real-Time PCR Detection system (Bio-Rad, California, US). Primer sequences are listed in the table below.

Table 3. Primers used in this study

Genes	Direction	Primer sequence
GAPDH	Forward	GTGGTCTCCTCTGACTTCAAC
	Reverse	ATTCGTTGTCATACCAGGAAATG
CAR	Forward	TGGCATGAGGAAAGACATGATAC
	Reverse	GATCAGCTCTTCTTGCTCCTTAC
CYP2A6	Forward	TTTTGGTGGCCTTGCTGGT
	Reverse	GGAGTTGTACATCTGCTCTGTGTTCA
CYP2B6	Forward	AAGCGGATTTGTCTTGGTGAA
	Reverse	TGGAGGATGGTGGTGAAGAAG
UGT1A1	Forward	GGTGACTGTCCAGGACCTAT
	Reverse	TAGTGGATTTTGGTGAAGGCAGTT
CD24	Forward	GCACTGCTCCTACCCACGCAGATTT
	Reverse	GCCTTGGTGGTGGCATTAGTTGGGT
CD44	Forward	CATAGAAGGGCACGTGGTGAT
	Reverse	ATACTGGGAGGTGTTGGATGTGA
CD133	Forward	CACTACCAAGGACAAGGCGTTC
	Reverse	CAACGCCTCTTTGGTCTCCTTG
EpCAM	Forward	AATCGTCAATGCCAGTGTACTT
	Reverse	TCTCATCGCAGTCAGGATCATAA

2.5 Western Blot

CAR, CYP2A6, CYP2B6 and UGT1A1 expression was examined by Western blot. Cells were lysed with RIPA buffer supplemented with protease inhibitor and dithiothreitol (DTT). In brief, total proteins from treated cells were extracted and protein concentration determined using the DC Protein Assay (Bio-Rad, California, US). Then resolved on SDS-polyacrylamide gels (8%) then transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% skim milk/TBST solution (tris-buffered saline, 0.1% Tween 20) for 1 hour, followed by incubation with primary antibodies at 4^{o}C. Membranes were incubated with horseradish peroxidase secondary antibodies for 1 h. Blots were developed using the West Pico and Femto chemiluminescent substrates (Thermo Fisher Scientific, Waltham, Massachusetts, US). Protein bands were quantified with Image J (National Institutes of Health). All antibodies and dilutions used in this study are listed in Table 4.

Table 4. Reagents for Western blot used in this study

Antibody/Reagent	**Source**	**Dilution**
Anti-Constitutive androstane receptor antibody	ab186869, Abcam	1:500
Anti-CYP2A6 antibody	ab3570, Abcam	1:1000
Anti-Cytochrome P450 2B6/CYP2B6 antibody	ab198870, Abcam	1:200
Recombinant Anti-UGT1A1 antibody [EPR9592]	ab170858, Abcam	1:1000
Recombinant Anti-Vinculin antibody [EPR8185]	ab129002, Abcam	1:20000
Goat Anti-Rabbit IgG H&L (HRP)	ab6721, Abcam	1:10000
PageRuler™ Prestained Protein Ladder, 10-180 kDa	26617, Thermo Fisher	

2.6 Immunohistochemistry

Liver cancer tissues and matched adjacent non-cancer liver tissues were obtained from patients undertaking liver resection in Westmead Hospital and Norwest Private Hospital. The project was approved by the Human Ethics Committee of WIMR [HREC/18/WMEAD/5 (5522)] and all patients provided written informed consent. Tissue samples were fixed with 10% neutral buffered formalin for 24 hours then embedded into paraffin blocks. A microtome was used to cut the paraffin blocks into sections of 4uM thickness and baked for 2 hours at 65°C onto glass microscopy slides. Slides were dewaxed and antigen retrieval was performed using a sodium citrate buffer (pH 6.0) for 15 minutes. Primary antibody was added at a dilution of 1:100 and incubated overnight at 4°C. Then secondary antibody (1:1000) was added and incubated for 1 hour at room temperature. Slides were placed in 3,3'Diaminobenzidine substrate for 10 minutes then counterstaining was performed using hematoxylin. Slides were rinsed then mounted with VectaMount® AQ Aqueous Mounting Medium (H-5501-60, Vector Laboratories, California, US). Slides were visualised using Hamamatsu NanoZoomer HT (Shizuoka, Japan) and a random field of view was taken and visualised at 20x magnification.

2.7 Functional assays

2.7.1 Proliferation Assay

The above-treated cells were seeded into 96-well plates (3000 cells/well) and maintained for 48 h. Cell counting kit-8 (CCK-8; Sigma-Aldrich) was used to measure the proliferation rate by optical density (OD)/absorbance using SpectraMax iD5 Microplate Reader (Molecular Devices, California, US) at 450 nm.

2.7.2 Transwell migration and invasion assay

Transwell cell culture inserts (diameter 6.5 mm, pore size 9 mm; Corning, New York, US) were placed into wells with (for migration) or without (for invasion) Matrigel (200 mg/mL;

Corning). Complete growth medium (supplemented with 10% FBS) was added to the bottom of the well prior to placing the inserts in the wells. Cells were resuspended in their respective media without FBS and seeded into the inserts and incubated for 24 h (migration) and 48 h (invasion) in a 5% CO_2 incubator at 37°C. Inserts were washed with PBS then fixed with ice-cold methanol for 15 min. Inserts were stained with 1% crystal violet (Sigma-Aldrich) for 25 min and washed with distilled water. Cells were visualised under microscope and 5 representative fields were photographed at 4x magnification for analysis. Images were analysed with Image J and total area occupied was quantified using the colour threshold tool.

2.7.3 Tumour sphere formation assay

Cell lines were cultured in ultra-low attachment plates (Sigma-Aldrich) in a 1:1 mixture of DME/F-12 (Thermo Fisher Scientific) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin (Thermo Fisher Scientific), 20 ng/ml human EGF recombinant protein (Thermo Fisher Scientific), human FGF-basic recombinant protein (Thermo Fisher Scientific), 2% B27 supplement minus vitamin A (Thermo Fisher Scientific), and 1% N-2 supplement (Thermo Fisher Scientific). Tumour spheres were cultured for 4 weeks with medium changed every 3~4 days, in the presence or absence of CITCO. Tumour spheres were stained with Hoechst 33342 and visualised with ChemiDoc MP (Bio-Rad, California, US). Images were further analysed with Image J. tumour spheres were collected for RNA extraction and cDNA was synthesized for subsequent qPCR analysis.

2.7.4 Cell cycle analysis

Cells were fixed with 70% ethanol at 4^{o}C for 1 hour, washed with PBS solution (PBS, 2mM EDTA and 1% FBS), stained with DAPI (0.1 µg/ml) and analysed with BD LSR Fortessa Cell Analyser (BD Biosciences, New Jersey, US). Data was analysed using FlowJo™ v10.8 Software (BD Biosciences).

2.8 Data analysis and visualisation

All statistical analysis was performed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California, USA). Difference in mRNA expression between any two groups was analysed by unpaired two-tailed *t*-test or paired *t*-test as appropriate. Ordinary one-way ANOVA tests followed by Tukey's multiple comparisons test were used to test the differences among the multiple independent groups. For survival data analysis, the two groups were compared using the Kaplan-Meier method and log-rank test. For all other differences in gene expression, results for functional assays, the mean and standard error of mean (SEM) was calculated. A p value of ≤ 0.05 is considered statistically significant.

Chapter Three

Exploring the role of CAR as a tumour suppressor by extensive biological functional assays

Abstract

HCC is one of the leading causes of cancer related deaths worldwide and has become a significant global health care challenge. Development of primary liver cancer can be driven and influenced by many risk factors comprising viral infections, lifestyle-based factors (e.g., heavy alcohol consumption, cigarette smoke), dietary factors, environmental and other underlying diseases. These all drive hepatocarcinogenesis without symptoms until the later stages of cancer progression. The classical "curative therapies" including surgical resections, local ablation and liver transplantation can only be applied to less than 20% of HCC patients since most of the HCC patients are diagnosed at very late stage, when no effective treatments are available. Hence, a constant unmet clinical need is to search the more effective therapies for this malignancy. This requires extensive studies into the molecular mechanisms of how HCC develops and why the existing treatments are ineffective.

Liver tumorigenesis involves aberration of a wide range of molecular pathways and their interactions, such as Notch pathway, Wnt pathway, and Signal transducer and activator of transcription 3 (STAT3) pathways while which pathway(s) is (are) more important may be dependent on the aetiology as well as the cellular, molecular, and genetic contexts. As such, targeting the key pathways involved in HCC pathogenesis may be efficacious for some patients but not in all and drug-resistant patients. The poor efficacy of the existing treatments on HCC is related to multifactorial factors but the presence of liver cancer stem cells (LCSCs) and heterogeneity of the HCC tumours play significant roles. Of relevance to this project, LCSCs are a key contributor to the drug resistance and treatment failure as this subset of liver cancer cells are highly oncogenic and refractory to therapy induced killing. As such, LCSCs are a potential therapeutic target for liver cancer therapy.

In this study we will investigate the role of CAR in human liver cancer at a more functional level using a variety of complementary assays. We attempt to delve deeper into the mechanism of action behind CAR as a tumour suppressor in HCC.

3.1 Introduction

CAR is a xenosensor that is almost exclusively expressed in the liver and regulates energy, lipid, and drug metabolism. CAR has been previously shown to be oncogenic in rodent liver tumorigenesis by inducing gadd45β, β-catenin, c-Myc activity, expression of CYP2B, cell proliferation, anti-apoptotic signaling and hepatic hypertrophy (22-29). However, more recently, *in vitro, and vivo* studies have shown that CAR exerts the opposite effect than its rodent counterpart (30-32). Epidemiologic studies have shown no link between liver cancer and prolonged phenobarbital, CAR agonist, treatment (33-35). Since then, multiple comparative studies have concluded that the mode of action for hepatocarcinogenesis in rodents and animal models cannot be applied humans (36, 37). Since then, other emerging roles of CAR have been studied by various groups. Studies have shown that CAR has a hepatoprotective role and that hepatic differentiation is driven by CAR (38). It has also been shown that CAR promotes stem cell differentiation and maturation (38). Interestingly, (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime (CITCO) treatment led to improved histological appearance, enhanced proliferative responses and better cell viability of human liver slices (38). These collectively suggest that CAR has a hepatoprotective role in the liver injury and regeneration process (38). Other than roles involved in the liver regeneration process, CAR was also revealed to play a tumour suppressive role in brain tumour stem cells (BTSCs) via regulating the activity CD133+ BTSCs and inhibited BTSC proliferation and expansion (15). A similar study has investigated the role of CAR in human liver cancer and have shown that CAR acts as a tumour suppressor by inhibiting erythropoietin signaling however, they did not use the human CAR specific agonist to activate CAR (39). Collectively, we can say that the mechanism of action underlying hepatocarcinogenesis in animal models cannot be applied to humans as there are clear species

differences. We can also conclude that CAR has a hepatoprotective role and acts as a tumour suppressor in human cancers including HCC which is the focus of this study.

Having successfully confirmed the knockdown and overexpression status in human HCC cell lines with the methodology outlined above, we then conducted a series of studies to investigate the biological roles of CAR in HCC cells, as reported below.

3.2 Investigating the role of CAR in HCC and LCSCs at a functional level

Constitutive androstane receptor (CAR) is a tumour suppressor in liver cancer and regulates stemness features

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Conflict of Interest Statement

The authors declare no conflict of interests.

Abstract

Constitutive androstane receptor (CAR) is a xenosensor that is almost exclusively expressed in liver. Studies in rodents suggest an oncogenic role for CAR in liver cancer, but its role in human liver cancer is unclear. This study investigated the functional roles of CAR in human liver cancer with a focus on the liver cancer stem cell (LCSCs) using siRNA, modulation of CAR activity by its specific agonist, and tumour sphere formation assays. CAR expression was significantly reduced in human liver cancer. Based on bioinformatic analyses and *in vitro* studies, activation of CAR significantly reduces cancer cell stemness and represses proliferation, migration, invasion, and the tumour sphere-forming abilities of liver cancer cells ($p<0.05$). CAR increased the expression of stemness markers CD24 and/or CD133 ($p<0.05$) in liver cancer cells. Collectively, CAR acts a tumour suppressor role in human liver cancer.

Key words: Constitutive androstane receptor (CAR), liver cancer, liver cancer stem cells (LCSCs), hepatocellular carcinoma (HCC), tumour suppressor, CITCO

Introduction

Constitutive androstane receptor (CAR) is a nuclear receptor (1) encoded by the gene NR1I3 (2). In humans, CAR is almost exclusively expressed in the liver (Human Protein Atlas available at: http://www.proteinatlas.org) with trace amounts detected in duodenum, brain, heart, and kidney (3). CAR is known for its role as a xenosensor but also plays important roles in normal liver physiology and liver regeneration, and in drug and energy metabolism, by regulating the transcription of target genes, including cyp2a6, cyp2b6, cyp3a4 and many more (4).

The role of CAR in tumorigenesis is controversial. As summarised by us (5), activation of CAR in mice enhances aberrant cell proliferation and facilitates hepatocarcinogenesis through activation of multiple pathways such as gadd45b, anti-apoptosis, β-catenin, c-Myc and CYP2B (6-9). However, activation of CAR in humans does not exert the same tumorigenic effects as in animal models (10-12). Thus, it has been proposed that the biological functions and mechanisms of action of CAR in the pathogenesis of liver cancer in mice may not be applicable to humans (13, 14). Hepatocellular carcinoma (HCC) is an aggressive cancer comprising a heterogeneous population of cancer cells including mature cancer cells and liver cancer stem cells (LCSCs), the latter a subset with strong self-renewing ability, differentiation potential, and enhanced refractoriness to drug-induced killing (15).

Recent studies suggest that CAR may be a tumour suppressor in human liver and brain cancers. For instance, activation of CAR by CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime), a human CAR-specific agonist, inhibited the growth and expansion of brain tumour stem cells (16). In a study on liver cancer, Hep3B and HepG2 cells with the tet-on system, and using soft agar colony assays and xenograft models, Li et al observed that CAR represses hepatocarcinogenesis by inhibiting erythropoietin

signalling (17). This study however did not investigate the role of xenobiotic-induced CAR activation, as has been done in rodents (17). Due to the species differences, such studies can only be performed in human cancer cells *in vitro* (5, 18-20).

We hypothesised that CAR acts as a tumour suppressor by regulating the stemness of liver cancer cells. We first undertook bioinformatics analyses of human HCC to explore the clinical relevance of CAR and then conducted a series of functional *in vitro* assays in multiple HCC cell lines and derived LCSCs to validate the tumour suppressor role of CAR in human HCC.

Materials and methods

Human tissues

Liver cancer tissues and matched adjacent non-cancer liver tissues were obtained from patients undertaking liver resection in Westmead Hospital and Norwest Private Hospital. The project was approved by the Human Ethics Committee of WIMR [HREC/18/WMEAD/5 (5522)] and all patients provided written informed consent.

Bioinformatics analysis of CAR expression and clinical implications

RNASeq data for CAR mRNA expression in human normal liver and HCC was downloaded from multiple GEO datasets (Supplementary Table 1). Data for patient overall survival (OS) and disease-free survival (DFS) and their respective hepatic NR1I3 mRNA expression levels were downloaded from GDC TCGA, TCGA, Firehose Legacy and GSE14520. Gene sets related to cancer prognosis, survival, recurrence, progression, proliferation, invasion, metastasis, angiogenesis, tumour immune response, stem cell features, signaling pathways involved in cancer growth and survival, as well as HCC specific gene sets were downloaded from Molecular Signatures Database V7.0 (MSigDB) and cBioPortal. Correlation between NR1I3 expression and gene sets was analyzed using Gene Expression Profiling Interactive Analysis 2 (GEPIA2) (Supplementary Table 2).

Data on the expression of NR1I3 and stem cell markers in liver cancer samples were downloaded from cBioPortal, MEXPRESS, GSE14520, GSE36376, GSE63898, GSE76297, GSE5975, GSE63898, GSE76297, GSE5975, GSE20238, GSE1898 and GSE76427. Correlation between NR1I3 and stem cell markers was assessed using GEPIA2 (Supplementary Table 3).

Detailed description of the bioinformatics analysis can be found in the Supplementary Materials and Methods.

Statistical analysis of online data

Detailed descriptions of the statistical analyses on the online data are available in Supplementary Materials and Methods.

Cell Culture

Three human HCC cell lines, Hep3B (passage 4), Huh-7 (passage 47) and PLC/PRF/5 (passage 4) were obtained from Cell Bank Australia. Hep3B and PLC/PRF/5 were cultured in Dulbecco's Modified Eagle Medium (DMEM; Lonza, 12-604F) supplemented with 10% FBS in a 5% CO_2 incubator at 37°C. Huh-7 was cultured in DMEM with low glucose (Sigma-Aldrich; D6046) supplemented with 10% FBS in a 5% CO_2 incubator at 37°C.

Activation of CAR in HCC cells

HCC cells were treated with the human CAR agonist CITCO (6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime). Briefly, cells were exposed to 1 mM of CITCO in growth media for the indicated time and DMSO was used as vehicle control. Effect of CITCO on CAR activation was confirmed by quantifying the gene expression of CAR specific downstream targets including CYP2A6, CYP2B6 and UGT1A1 by qPCR (Supplementary Figure 1)

Modulation of CAR expression in HCC cells

CAR expression in HCC cells was down-regulated by using a CAR specific siRNAs or overexpressed by using a plasmid carrying human CAR encoding NR1I3. Based on a series of titration assays in three HCC cell lines, the optimal concentration of CAR specific siRNAs (siCAR) (Dharmacon, USA) was determined to be 40 nM at which concentration, >60% knockdown of CAR expression was achieved (Supplementary Figure 2) and thus this concentration was used in subsequent studies. For knockdown studies, cells were seeded onto 6-well plates and grown till 70% confluency. Cells were then transfected with siCAR for 48 h prior to functional assays as described below.

To overexpress CAR, HCC cells were transfected with the expression plasmid containing human CAR encoding gene NR1I3 (pCMV6-Entry-NR1I3) (Origene Technologies, US) in Opti-MEM reduced-serum containing FuGene (Promega, Wisconsin, US). Cells were transfected for 48 h prior to functional assays. Successful knockdown and overexpression of CAR was confirmed by qPCR and western blot.

Quantitative real time RT-PCR (qPCR)

Total RNA was extracted using FavorPrep Tissue Total RNA Kit (Favorgen, Taiwan) according to the manufacturer's protocol, and was used to generate cDNA using M-MLV reverse transcriptase (Promega, Wisconsin, US). cDNA was diluted (1:10) and used for qPCR with QuantiNova SYBR Green PCR Kit (Qiagen, Netherlands). qPCR cycling conditions included an initial activation step (2 min, 95^{o}C), denaturation (5s, 95^{o}C) and combined annealing/extension (10s, 60^{o}C) for 35-40 cycles. qPCR was performed using the CFX96 Touch Real-Time PCR Detection system (Bio-Rad, California, US). All qPCR data was analysed using the double delta Ct analysis. Primer sequences are listed in Supplementary Table 4.

Western Blot

The expression of CAR was examined by Western blot. In brief, total proteins from treated cells were extracted, protein concentration determined, and resolved on SDS-polyacrylamide gels (8%) and transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% skim milk/TBST solution (tris-buffered saline, 0.1% Tween 20) for 1 h, followed by incubation with primary antibodies at 4^{o}C. Membranes were incubated with horseradish peroxidase secondary antibodies for 1 h. Blots were developed using the West Pico and Femto chemiluminescent substrates (Thermo Fisher Scientific). Protein bands were quantified with Image J (National Institutes of Health). All antibodies and dilutions used in this study are listed in Supplementary Table 5.

Proliferation Assay

Treated cells were seeded into 96-well plates (3000 cells/well) and maintained for 48 h. Cell Counting Kit-8 (CCK-8; Sigma-Aldrich, US) was used to measure the proliferation rate by optical density (OD)/absorbance using the SpectraMax iD5 Microplate Reader (Molecular Devices, CA, US) at 450 nm.

Transwell migration and invasion assay

Transwell cell culture inserts (diameter 6.5 mm, pore size 9 mm; Corning, USA) were placed into wells with (for migration study) or without (for invasion study) Matrigel (200 mg/mL; Corning, US). Complete growth medium (supplemented with 10% FBS) was added to the bottom of the well prior to placing the inserts in the wells. Cells were resuspended in their respective media without FBS and seeded into the inserts and incubated for 24 h (migration) and 48 h (invasion) in a 5% CO_2 incubator at 37°C. Inserts were washed with PBS then fixed with ice-cold methanol for 15 min. Inserts were stained with 1% crystal violet (Sigma-Aldrich, USA) for 25 min and washed with distilled water. Cells were visualised under the microscope

and 5 representative fields were photographed at 4× magnification for analysis. Images were analysed with Image J and total area occupied was quantified using the colour threshold tool.

Tumour sphere formation assay

Cells were cultured in ultra-low attachment plates (Sigma-Aldrich, CLS3471) in a 1:1 mixture of DME/F-12 medium supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin 20 ng/ml human EGF recombinant protein, human FGF-basic recombinant protein, 2% B27 supplement minus vitamin A and 1% N-2 supplement. Tumour spheres were cultured for 4 weeks with medium changed every 3~4 days in the presence or absence of CITCO. Tumour spheres were stained with Hoechst 33342 and visualised with ChemiDoc MP (Bio-Rad, USA). Images were further analysed with Image J. Tumour spheres were collected for RNA extraction and cDNA was synthesized for subsequent qPCR analysis.

Cell cycle analysis

Cells were fixed with 70% ethanol at 4ºC for 1 h, washed with PBS solution (PBS, 2mM EDTA and 1% FBS), stained with DAPI (0.1 µg/ml) and analysed with BD LSR Fortessa Cell Analyser. Data was analysed using FlowJo™ v10.8 Software (BD Life Sciences).

Results

CAR expression is downregulated in human HCC

As show in Figure 1, human HCCs express significantly less CAR as compared to adjacent non-HCC liver both at the mRNA (Figure 1, A) and protein (Figure 1, B) level. The reduced CAR expression pattern was also demonstrated by immunohistochemistry (Supplementary Figure 3) and in HCC cells (Figure 1, C). The reduced expression of CAR in HCC was associated with reduced levels of CAR downstream targets (Figure 1, D) indicating reduced CAR activity in human HCC. These data support our hypothesis that CAR plays a tumour

suppressor role in human HCC. Bioinformatics analysis from nine datasets (Supplementary Table 1) was then performed to investigate the clinical relevance. As shown in Figure 2A, CAR expression is significantly reduced in HCC as compared to the matched non-HCC liver in 8 out of 9 datasets. Bioinformatic analysis of microarray data, gene and protein expression analyses using patient tissue, collectively show the decrease in CAR expression in HCC tissue when compared to non-tumour tissue.

CAR is downregulated in patients with higher HCC grade and stage

We analysed the CAR expression pattern in relation to tumour stage (n=330) and grade (n=326) in GDC TCGA LIHC dataset (Figure 2, B, C). Progression of tumours from Stages 1 to 4 was associated with a decreasing trend in CAR expression with significant changes occurring between patients in Stage 1 and those in Stage 3 (p<0.0001). Patients with Grades 3 and 4 HCC showed the lowest level of CAR expression (p<0.01).

The expression of CAR in relation to tumour stages and grades were also analysed using data from the TCGA Firehose Legacy dataset (Figure 2, D, E). A decreasing trend of CAR was again observed from Stages 1 to 4 (p<0.05 in Stage 1 vs 3) and Grades 1 to 4.

Using data from the GSE89377 dataset, we analysed the expression of CAR in various precancerous liver conditions (Figure 2, F, G). Compared to normal subjects, patients with pre-cancerous liver diseases including chronic hepatitis, liver cirrhosis, dysplastic hepatic nodules, as well as those from Grades 1 and 2 HCCs, patients with Grade 3 HCC showed a significant reduction in CAR expression (p<0.001, compared to normal liver). When HCC patients were grouped into early and late HCCs, those with late HCC had a significantly lower level of CAR than those in the early stages (p<0.05). After thorough reviewing of microarray data, there is a significant pattern whereby CAR expression decreases as cancer grade and stage worsens.

Higher levels of CAR expression are associated with better survival

Kaplan-Meier analysis was used to assess the impact of CAR on overall survival (OS) and disease-free survival (DFS). As revealed in the TCGA Firehose Legacy dataset, high CAR is significantly associated with better OS [Figure 2, H; Hazard Ratio (HR), 0.5886; 95% Confidence Interval (CI): 0.2995-0.863; p=0.0152]. Compared to patients with low CAR expression, those with higher CAR expression generally have better 3- and 5-year OS rate (70% vs 43.7%, and 49.8% vs 28.5%, respectively). A similar trend was observed for OS in the GDC TCGA LIHC dataset *albeit* this was not significant (Figure 2, I). Similarly, HCC patients with higher CAR expression in GSE14520 dataset also showed a better 1-, 3- and 5-year OS rate than those with lower CAR expression (94.5% vs 83.5%, 82.7% vs 55.7%, and 74.1% vs 43.7%, respectively) (Figure 2, J, HR: 0.3870; 95% CI, 0.2528-0.5924; p<00001). In addition, HCC patients with higher CAR expression in the GSE14520 dataset showed a significantly better DFS than those with low CAR expression (Figure 2, K, HR, 0.5411; 95% CI, 0.3788-0.7731, p=0.0012). Kaplan-Meier analyses show that patients with higher levels of CAR have a better overall and disease-free survival when compared to patients expressing lower levels of CAR.

CAR expression correlates with genes involved in cancer development and drug resistance

The correlation between CAR expression level in HCC with that of the genes involved in invasion, metastasis, cancer stem cell phenotype, HCC progression and disease prognosis was assessed. As shown in Figure 3, A, CAR negatively correlated with the genes involved in invasion and metastasis as defined by cBioPortal (r=-0.52, p<0.0001). A moderate positive correlation was seen between CAR expression and vascular invasion related genes (Minguez: genes down-regulated in HCC with vascular invasion, r=0.59, p<0.0001). CAR negatively

correlated to genes over-expressed in the proliferative subclass of HCC (Chiang: genes over-expressed in the "proliferation" subclass of HCC, r=-0.52, $p<0.0001$) but positively correlated with the genes that are down-regulated in the proliferative subclass of HCC (Chiang: genes down-regulated in the "proliferation" subclass of HCC, r=0.68, $p<0.0001$). A moderate negative correlation was observed between CAR expression and genes down-regulated during the transition from G2 to G3 (Iikuza: genes upregulated in G3 compared to G2, r=-0.57, $p<0.0001$).

In HCC patients with poor survival, CAR expression negatively correlated with the over-expressed genes (Kim: genes over-expressed in HCC with poor survival, r=-0.59, $p<0.0001$) whereas it positively correlated with the down-regulated genes (Kim: genes under-expressed in HCC with poor survival, r=0.58, $p<0.0001$). In HCC patients with poor survival, CAR expression negatively correlated with the over-expressed genes (Lee: genes highly expressed in HCC with poor survival, r=-0.5, $p<0.0001$) whereas in the HCC patients with good survival, CAR expression positively correlated with the over-expressed genes (Lee: genes highly expressed in HCC with good survival, r=0.63, $p<0.0001$). CAR expression also positively correlated with the favorable prognostic genes from The Human Protein Atlas (r=0.55, $p<0.0001$). Overall, results show that CAR is positively correlated to gene signatures that are upregulated in patients with better survival, disease progression and prognosis or with markers that are suppressed or downregulated in processes that define a worse disease progression or status.

CAR expression correlates with stem cell markers in HCC

A moderate positive correlation was seen between CAR expression and genes down-regulated in $EpCAM^+$ HCC (Yamashita: genes down-regulated $EpCAM^+$ HCC only, r=0.56, $p<0.0001$) and genes down-regulated in HCC cells that are related to hepatic stem cell properties

(Yamashita: genes down-regulated in HCC cells with hepatic stem cell properties, r=0.61, p<0.0001) (Figure 3, A).

The association between CAR expression and the expression pattern of stemness markers in HCC samples was analysed by Spearman's Correlation analysis. A general trend of negative association between CAR and the most reported stemness markers in HCC (CD24, CD44, CD133, EpCAM) was identified (Figure 3, B). More specifically, there is a moderate negative correlation between CAR and CD24 in 50% (5 out of 10) of the datasets (r<-0.5, p<0.05). CAR expression in the TCGA, Firehose Legacy shows the strongest negative association with CD24 compared to all other datasets (r=-0.58, p<0.05). In GSE76297, CAR has a moderate negative correlation with EpCAM and CD133 (r=-0.58, -0.51, respectively, p<0.05). Weaker negative associations between CAR expression and other stemness markers were seen across all datasets although not statistically significantly (Supplementary Table 3). After detailed analyses of gene expression of stemness markers from ten datasets, results show a significant negative correlation between CAR and CD24, CD44, CD133 and EpCAM in HCC tissue.

Functional roles of CAR in HCC cells

CAR activation hinders proliferation, migration, invasion, and sphere-forming ability of HCC cells

To investigate the biological functions of CAR in the pathogenesis of HCC, we activated CAR in HCC cells using CITCO, a human CAR specific agonist. The effect of CAR activation on cell proliferation was assessed by CCK-8. Activation of CAR significantly decreased the proliferation (Figure 4, A), migration and invasion (Figure 4, B, C) of all three cell lines (Figure 4, B, C). CITCO treatment also decreased tumour sphere formation consistently across three cell lines (Figure 4, D). RNA was extracted from these tumour spheres and expression of major stem cell markers were studied. CITCO treatment decreased the expression of all stem cell

markers in the tumour spheres derived from Huh-7 and PLC/PRF/5 cells (Figure 4, E), while only CD44 and EpCAM expression was significantly reduced in the CITCO treated tumour spheres derived from Hep3B cells (Figure 4, E). Meanwhile, an acute increase in the percentage of cells in the S phase following CITCO treatment together with a concomitant decrease in the percentage of cells in the G1 phase was observed (Supplementary Figure 4), although these changes did not reach statistical significance. CITCO treatment decreased HCC cell line proliferation, migration, invasion and tumour sphere formation along with a significant decrease in stemness markers.

CAR is essential in driving the functional aspects of liver cancer cells

To clarify the roles of CAR in HCC, we used a CAR over-expressing plasmid. Over-expression of CAR reduced the proliferation of Huh-7 cells ($p<0.05$) but not the other two cell lines (Supplementary Figure 5, A). Meanwhile, CAR over-expression did not alter the migration and invasion of all three cell lines (Supplementary Figure 5, B, C).To further elucidate the biological roles of CAR in HCC, CAR expression was knocked down using CAR siRNA (siCAR). As shown in Figure 5A, knockdown of CAR significantly increased proliferation of Hep3B (by 1.6-fold), Huh-7 (by 1.9-fold) and PLC/PRF/5 (by 1.4-fold) cells ($p<0.01$). As shown in Figure 5B, C, knockdown of CAR also significantly increased migration of Hep3B (by 1.7-fold), Huh-7 (by 2-fold) and PLC/PRF/5 (by 2.1-fold) cells. Additionally, CAR knockdown resulted in a significant increase in invasion of Hep3B and Huh-7 cells and a similar trend was observed in PLC/PRF/5 cells.

We examined the effect of CAR knockdown on cell cycle progression. CAR knockdown significantly decreased the proportion of cells in the G1 phase of Hep3B (by 14%), Huh-7 cells (by 40%) and PLC/PRF/5 (by 22%) cells, with a concomitant increase in the proportion of cells seen in S and G2 phases in Huh-7 (by 22% and 17%, respectively) and PLC/PRF/5 cells (by

14% and 8%, respectively) (Figure 5, D, $p<0.05$). A trend of increase in percentage of cells in S and G2 phase was observed in Hep3B cells. Whilst CAR overexpression did not yield significant results, knockdown of CAR resulted in a significant increase in proliferation, migration and invasion of HCC cells.

CAR regulates the expression of stem cell markers

To study the impact of CAR on the expression of liver cancer stem cells, we modulated CAR by activation (using CITCO, in HCC cells and tumour spheres derived from HCC cells) and by CAR knockdown (using siCAR, in HCC cells). Based on bioinformatics analyses (Figure 3, B), CAR is significantly negatively correlated with the expression level of several key markers of cancer stem cells including CD24, CD44, CD133 and EpCAM. Thus, we verified the regulatory effect of CAR on stemness features. CAR activation by CITCO significantly decreased CD24 expression in Hep3B and PLC/PRF/5 cells with a decreasing trend seen in Huh-7 cells (Supplementary Figure 6, A). The impact of CAR activation on the expression of these stem cell markers was even more prominent in the tumour spheres, where CITCO significantly reduced the expression of multiple cancer stem cell markers including CD44 and EpCAM (in the tumour spheres derived from all three cell lines), CD24, CD44, CD133 and EpCAM (in the tumour spheres derived from Huh-7 and PLC/PRF/5 cells) (Figure 4, E). Knockdown of CAR resulted in increased expression of the above four cancer stem cell markers in all three HCC cell lines although only significant for CD133 in PLC/PRF/5 cells (Supplementary Figure 6B). We found that CITCO treatment suppressed stemness marker expression and CAR knocked-down cells showed the opposite phenomenon.

Discussion

CAR is almost exclusively expressed in liver. Thus, deciphering its precise function could potentially lead to the discovery of a liver-specific target for therapy of liver disease. Using bioinformatic analyses we show that CAR expression is reduced in HCC datasets, a finding that was verified in our tissue bank. Lower expression of CAR correlated with poor prognosis by gene correlation and survival analyses. CAR expression negatively correlated with the expression of the stem cell markers CD24, CD44, CD133 and EpCAM, which were also decreased *in vitro* following CITCO treatment. In functional assays, CAR activation reduced cancer cell proliferation, migration, and invasion; these effects were reversed by siRNA-mediated CAR knockdown. Finally, CAR activation impacted stemness features of HCC cells leading to reduced expression of the stem cell markers including CD24 and impaired the ability to form tumour spheres.

Cancer stem cells are identified using cell surface markers and although no markers are exclusively specific to LCSCs (15), we identified CD24, CD44, CD133 and EpCAM that are correlated to CAR expression. A novel finding is that CAR activity impacts the functionality of liver cancer stem cells. CAR activation (by CITCO) decreased the stemness of these cells with decreased expression of CD24 and CD133, and this was reversed by siCAR. Importantly, the effects on stemness features were recapitulated in tumour spheres derived from these cells following CAR activation. Our results support the hypothesis that CAR acts as a tumour suppressor by its impact impacts on LCSCs. It is established that CD24 and CD133 are involved in propagating uncontrolled proliferation, migration, and invasion in HCC (21-24). Therefore, as expected, repression or downregulation of these markers by CAR activation, attenuated cancer growth and migration. Consistently, a previous study found that CITCO activation of CAR resulted in a decrease in $CD133^+$ brain tumour stem cells (16).

Uncontrolled cell proliferation as well as increased migratory and invasive capabilities are key features of malignant cells. In rodent models, CAR stimulates cell proliferation and promotes liver regeneration and thus promotes cancer formation (25-28). By contrast, in this study, activation of CAR resulted in a reduction in the proliferation, migration, and invasion of human HCC cells, consistent with a tumour suppressive role. The inhibitory effects of CAR were reduced by siRNA-mediated CAR knockdown which resulted in a concomitant increase in the proportion of cells in the S and G2 phases of the cell cycle. These functional readouts are supported by bioinformatics analyses where decreasing CAR expression was seen in more advanced and later stage HCCs. Interestingly, forced CAR overexpression, unlike that by Li et al (2022) (17), did not impact proliferation, migration, or invasion. This suggests that activation of CAR activity but not merely its overexpression is required for the tumour suppressive functions. Our findings support a need for further studies on the role of CAR activators as an approach to liver cancer therapy.

In bioinformatic analyses, a lower expression of CAR correlated with worse prognosis and HCC progression, while its expression was reduced in advanced grade and stage HCC. This suggests that inducing CAR activity by xenobiotics in advanced HCC might attenuate cancer progression.

Whilst the species differences in CAR function limit us to *in vitro* studies, our data demonstrate an unequivocal tumour suppressive role of CAR in liver cancer. While more detailed mechanistic studies are warranted, the efficacy of CAR xeno-activators in the treatment of advanced HCC warrant further study.

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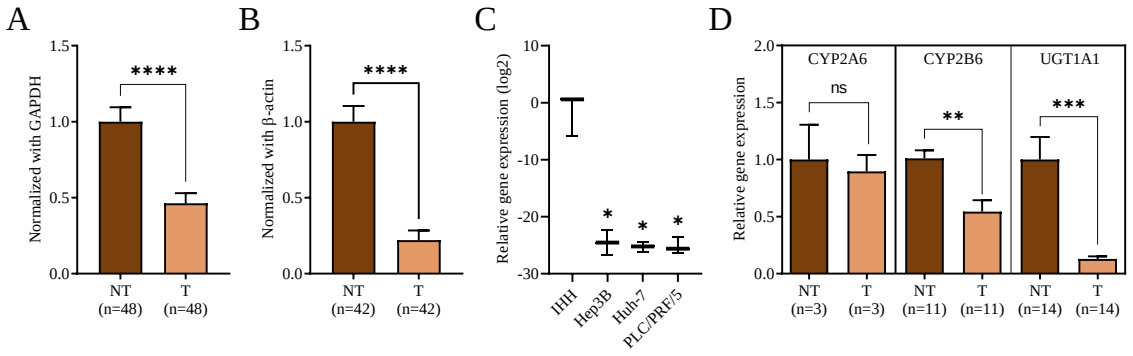


Figure 1. **CAR expression patterns in human HCC**. CAR expression was detected at the mRNA (**A**) and protein (**B**) levels in human HCC tissues and matched non-HCC tissues using western blot and densitometry. The expression of CAR in established HCC cell lines was examined by qPCR (n=3), using an immortalized normal human hepatocyte line (IHH) as a control (**C**). The expression of CAR specific downstream targets including CYP2A6, CYP2B6 and UGT1a1 (**D**) in the same tissues as in Panels A and B was examined by qPCR. T: HCC tumours; NT: matched non-HCC tumours. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$. ns: not significant.

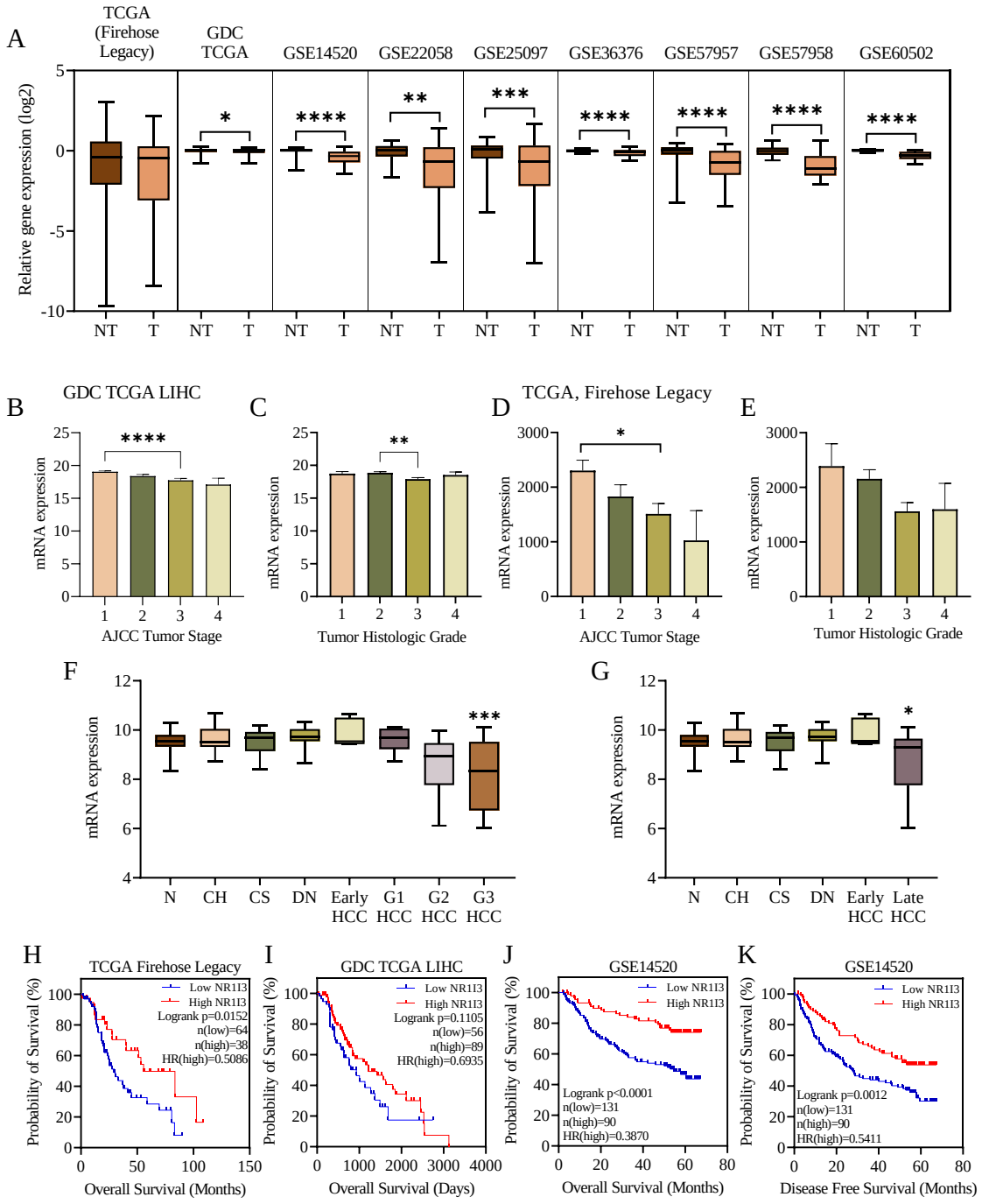


Figure 2. Correlation of CAR expression with HCC clinical features. A. Bioinformatic analysis of CAR expression in HCC tissues and matched non-HCC tissues. B-E: CAR expression in different stages and histologic grades of HCC tissues in GDC TCGA LIHC (B, C) and TCGA, Firehose Legacy (D, E) datasets. Association of CAR expression with HCC grades and stages was further verified in GSE89377 dataset (F, G), where a significant reduction of CAR was seen only in more advanced and late-stage HCC (F) and more

specifically in G3 and 4 HCC (G) but not in various pre-cancerous conditions. Patients with higher CAR expression (high NR1I3) showed better OS in TCGA Firehose Legacy (H), GDC TCGA LIHC (I) and GSE1450 (J) datasets. A better DFS was also seen in patients with higher CAR expression (K). T: HCC tumours; NT: matched non-HCC tumours; N: normal liver; CH, chronic hepatitis; CS, cirrhosis; DN, dysplastic nodule; G1-3: grades 1-3 HCC. HR, hazard ratio. *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001.

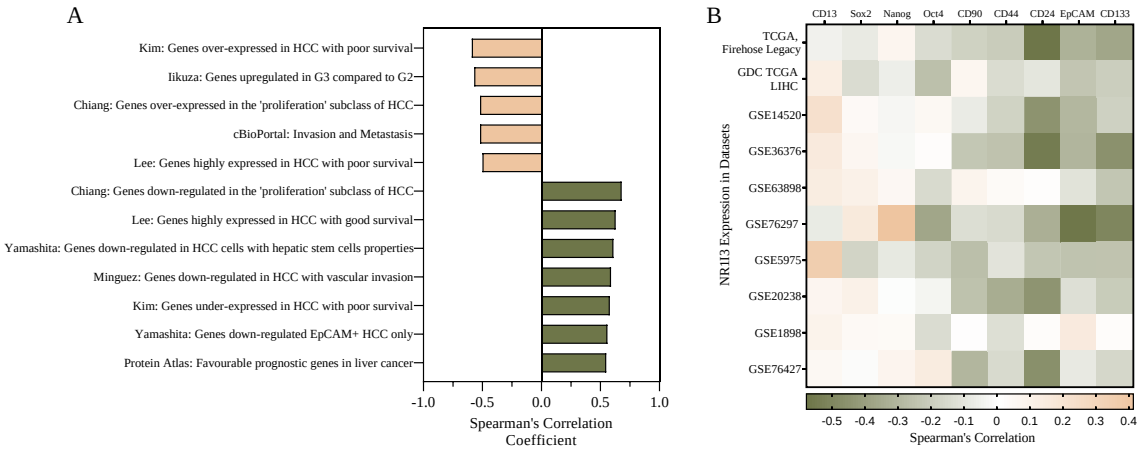


Figure 3. Correlation between CAR expression, gene signatures and stem cell markers. Gene signatures were downloaded from MSigDB, the Human Protein Atlas and cBioPortal. Spearman's correlation analysis was performed using GEPIA2. CAR negatively correlated to genes that are over-expressed or involved in HCC growth and progression while the opposite trend was discovered with the gene signatures that are downregulated in high grade HCC and those associated with better HCC survival (p<0.05) (A). A significant negative correlation between CAR and CD24, CD44, CD133 and EpCAM can also be seen in the heatmap (p<0.05) (B).

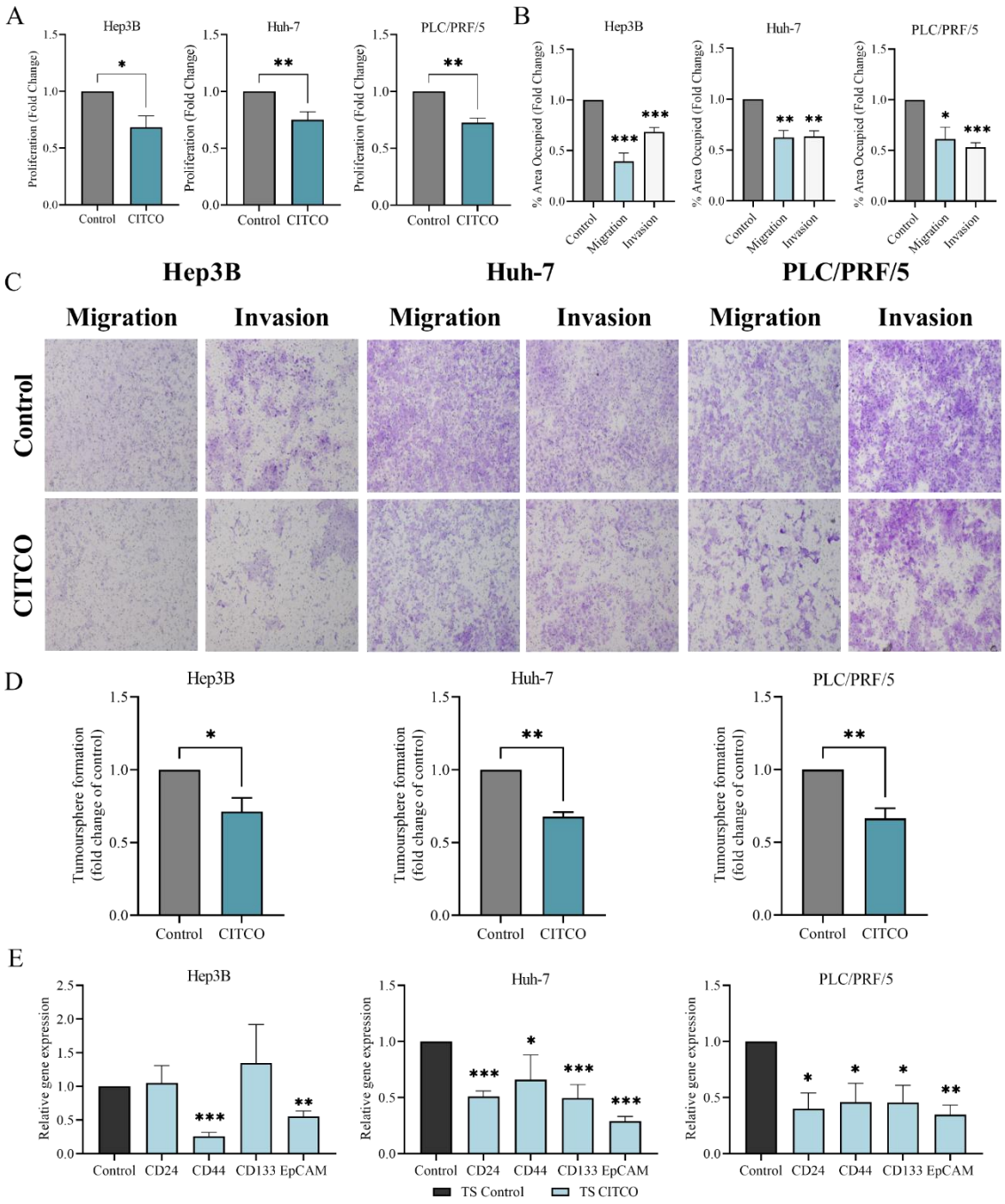


Figure 4. CAR activation decreased proliferation, migration, invasion, and tumour sphere formation of HCC cells. HCC cells were pre-treated with 1 mM of CITCO for 48 h, and then further cultured in the presence of 1 mM of CITCO for 24 h (for migration assay) or 48 h (for proliferation and invasion assay). A significant decrease in the proliferation (**A**), migration and invasion (**B**) was seen in Hep3B, Huh-7 and PLC/PRF/5 cells. **C.** Representative images of migration and invasion of HCC cells treated with CITCO. **D.** Quantitative analysis

of the tumour sphere formation assay showing decreased sphere-forming ability in CITCO treated cells. **E.** Significant reductions in the gene expression of stem cell makers in CITCO treated tumour spheres. *: $p<0.05$; **: $p<0.01$; ***$p<0.001$. All data were derived from three independent experiments each with three individual tests and are expressed as mean ± SEM.

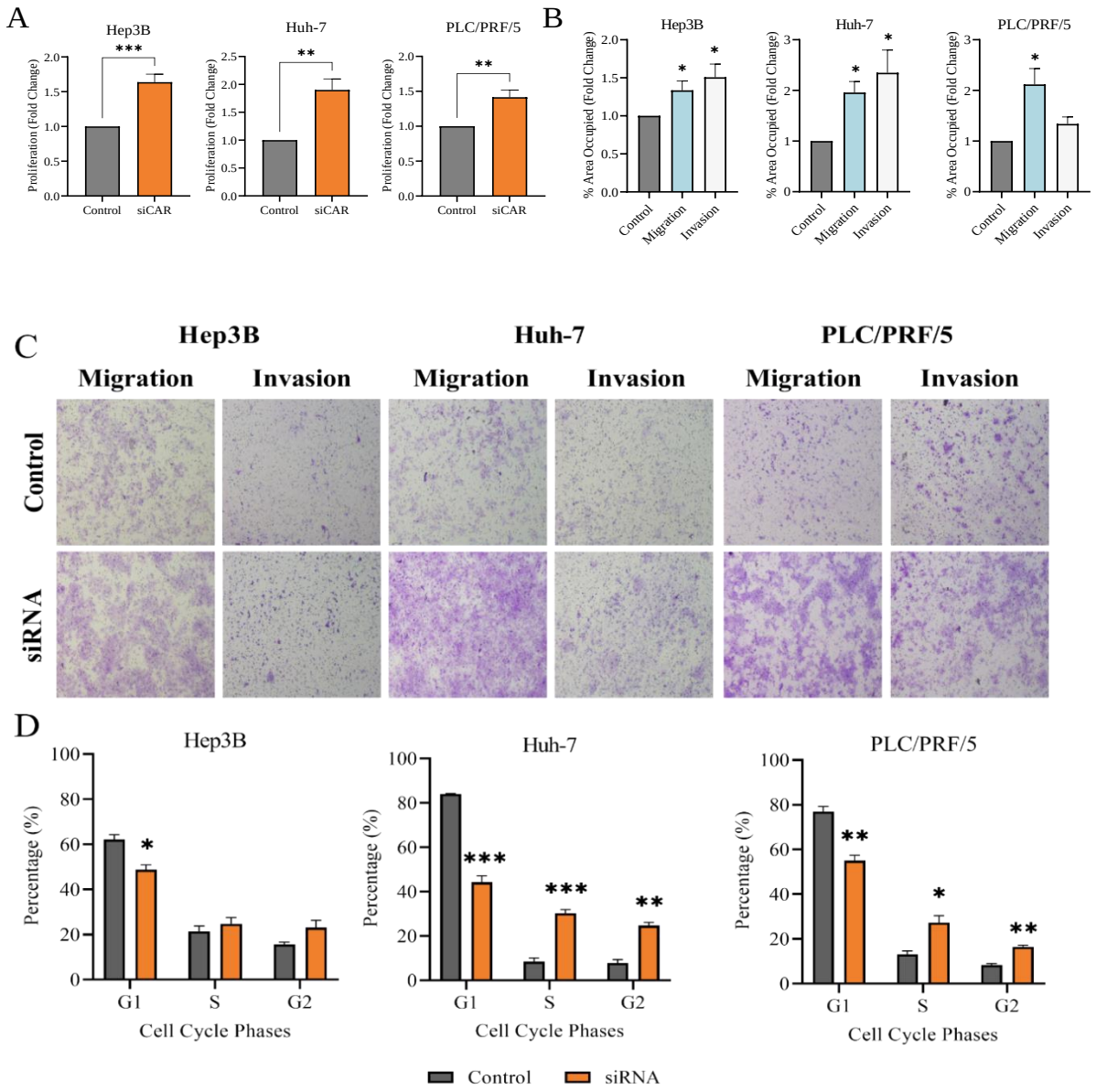
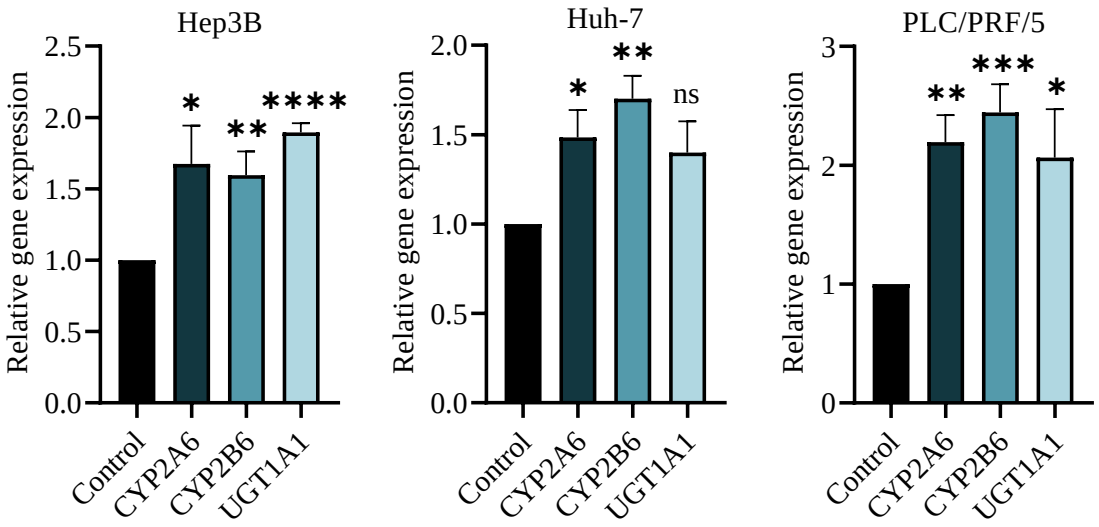
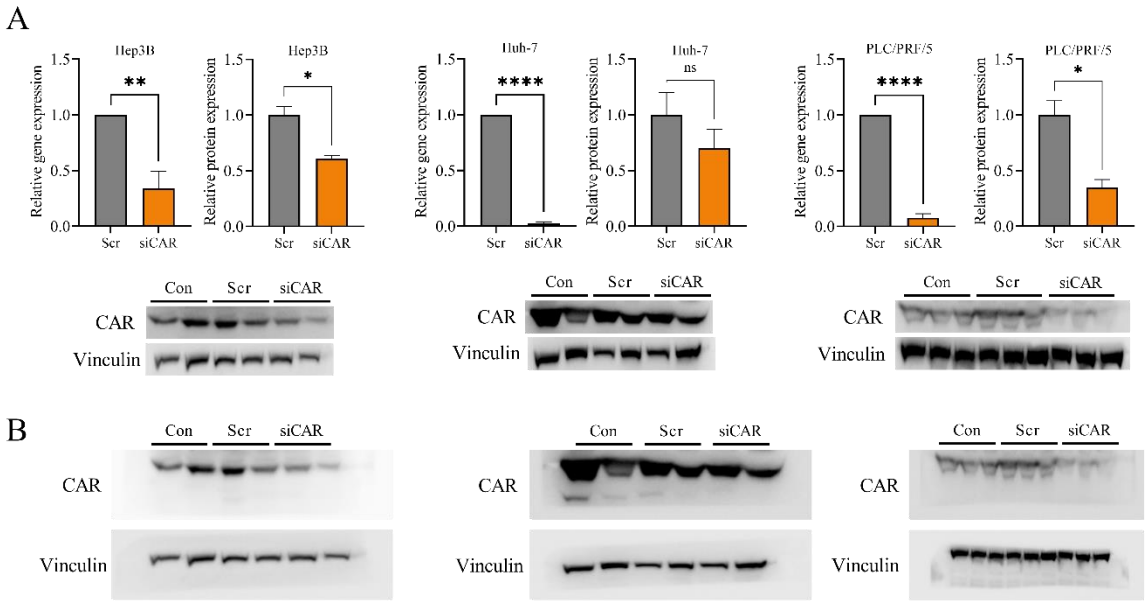


Figure 5. Effects of CAR knockdown on the proliferation, migration, invasion, and cell cycle distribution of HCC cells. Transient CAR knockdown by siCAR led to a significant increase in the proliferation of HCC cells (**A**). CAR knockdown also enhanced migration and invasion across all three cell lines (**B**). **C.** Representative images of migration and invasion of HCC cells with CAR knockdown. **D.** CAR knockdown resulted in a decreased proportion of cells in G1 phase and a concomitant increase in the proportion of cells in S and G2 phases. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. All data were derived from three independent experiments each with three individual tests and are expressed as mean ± SEM.

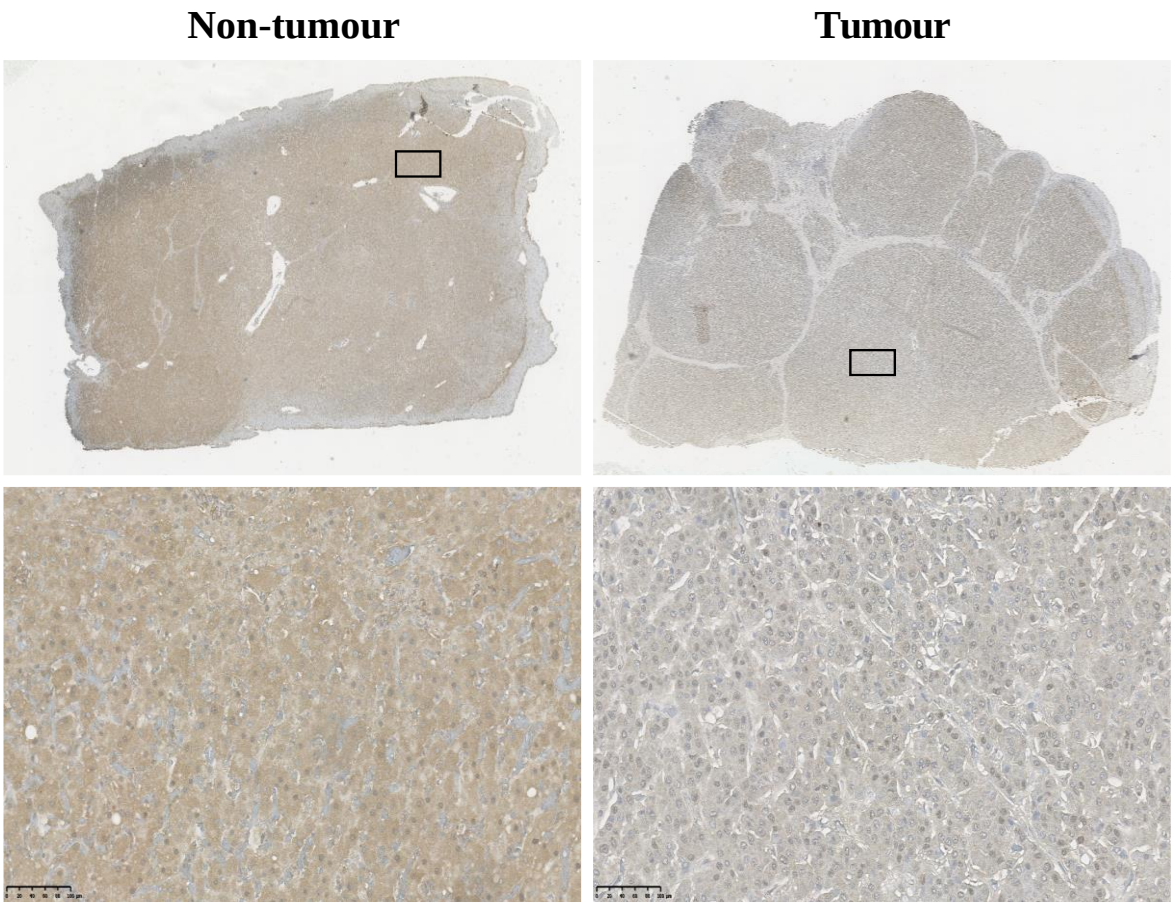
Supplementary Figures



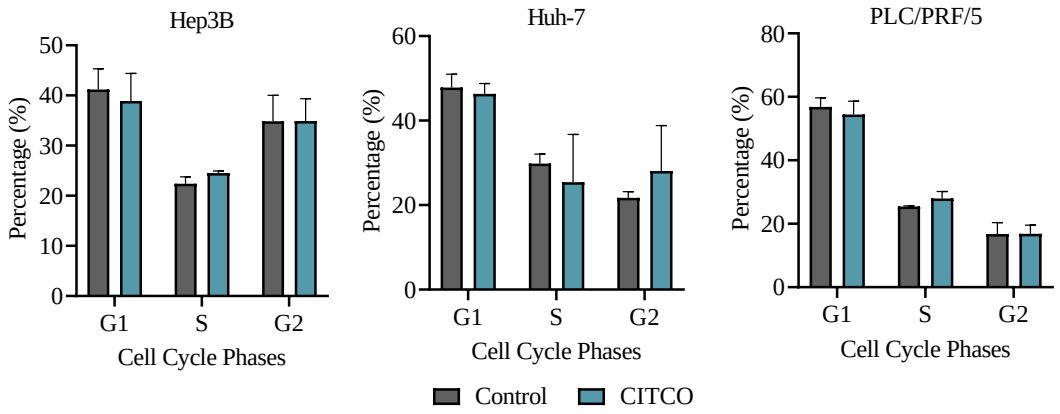
S. Figure 1. Successful CAR activation in HCC cells. (A) HCC cells were treated with 1µM of CITCO. The expression of the downstream targets was examined 48 h later at the mRNA (n=3 for all) by qPCR. Control: Vehicle control; *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001; ns: not significant. **S. Figure 1. Successful CAR activation in HCC cells. (A)** HCC cells were treated with 1µM of CITCO. The expression of the downstream targets was examined 48 h later at the mRNA (n=3 for all) by qPCR. Control: Vehicle control; *: p<0.05; **: p<0.01; ***: p<0.001; ****: p<0.0001; ns: not significant.



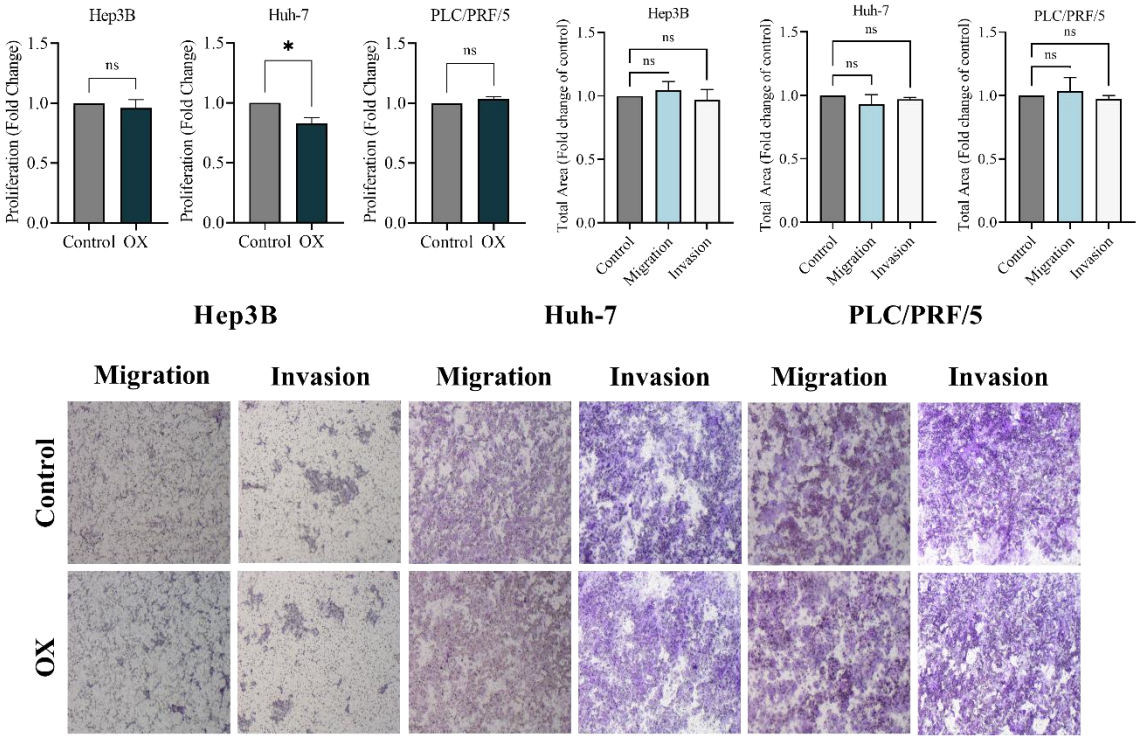
S. Figure 2. Successful CAR knockdown in HCC cells. (A) HCC cells were transiently transfected with 40 nM of CAR siRNA (siCAR). The expression of CAR was examined 48 and 72 h later at the mRNA (n=3 for all) and protein levels (n=2 for Hep3B and Huh-7 and n=3 for PLC/PRF/5) by qPCR and Western blots, respectively. **(B)** Un-Cropped images of original western blots shown above. siCAR: cells transfected with CAR siRNA; Scr: cells transfected with scrambled siRNAs. Control: naïve cells. *: $p<0.05$; **: $p<0.01$; ****: $p<0.0001$; ns: not significant.



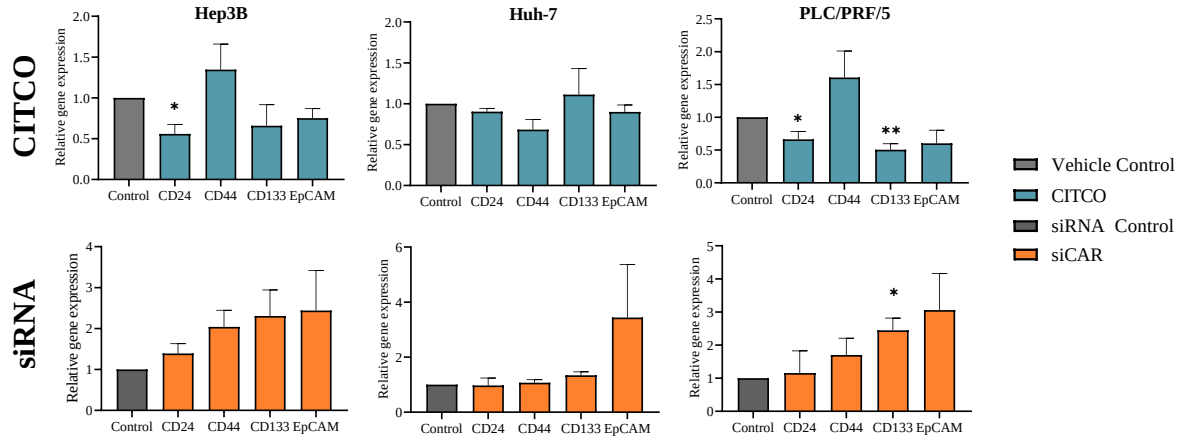
S. Figure 3. Immunohistochemistry reveals a lower tissue wide expression of CAR in tumour when compared to non-tumour tissue. Immunohistochemistry was performed and slides were scanned then a random field of view was selected for visualisation at 20X (n=1). Data is preliminary but show a decreasing pattern whereby tumour tissue shows a lower expression of CAR.



S. Figure 4. Cell cycle analysis in HCC cells treated with CITCO. CITCO-treated cells were analysed with flow cytometry. A decreasing trend in the percentage of cells in G1 phase and a parallel increase in the percentage of cells in S phase can be seen. Data were derived from 3 separate experiments each with 3 replicates.



S. Figure 5. Effect of CAR over-expression on the proliferation, migration, and invasion of HCC cells. HCC cells were transiently transfected with CAR over-expressing plasmid. After 48 h of transfection, cells were assayed for proliferation (**A**), migration and invasion (**B, C**). CAR overexpression only mildly inhibited cell proliferation of Huh-7 cells but not other cells (**A**). Transient over-expression of CAR showed no effects on the migration and invasion of all 3 HCC cell lines (**B, C**). Representative images of migration and invasion studies are shown. OX: over-expression. *: $p<0.05$; ns: not significant.



S. Figure 6. Effects of CAR modulation on the expression of cancer stem cell markers. A. Effect of CAR activation by CITCO on the expression of cancer stem cell markers in HCC cells. **B**. Effect of CAR knockdown by siCAR on the expression of cancer stem cell markers in HCC cells. Expression of these markers was examined by qPCR. TS, tumour spheres. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

Chapter Four

General discussion and future directions

4.1 Discussion

CAR is almost exclusively expressed in the liver (40). CAR is involved in many biological processes and even implicated in rodent hepatocarcinogenesis (9). However, recent studies, including the current study, have shown otherwise. Based on our extensive bioinformatics analyses and our wet laboratory studies, we hypothesized that CAR is a potential tumour suppressor in the pathogenesis of liver cancer formation. In this project, we aimed to unveil the potential mechanism of action or key signalling pathway involved in CAR activation that inhibits biological functioning of LCSCs and liver cancer cells.

HCC has a heterogeneous population of cells including liver cancer stem cells (41). The functional characteristics of an aggressive tumour like HCC includes uncontrolled proliferation, increased migratory and invasive phenotype as well as increased LCSC growth and expansion (41). Previous studies have shown that CAR is oncogenic and drives rodent tumorigenesis (25, 28, 29, 42, 43). Soon later, it was concluded that the rodent mechanism of action cannot be applied to humans as many studies were unable to find a significant link between human CAR and human liver carcinogenesis (30, 33, 34, 36, 44-47). In the current study, we have demonstrated that activation of CAR using a human CAR specific agonist, CITCO, repressed proliferation, migration and invasion of HCC cells. Further, CAR activation of CAR impaired the stemness of liver cancer cells. These findings are in line with previous studies whereby activation of CAR decreased growth and expansion of $CD133^{+}$ brain tumour stem cells (15) and CAR activation led to a decreased proliferation of HCC cells via inhibiting the activity of hepatic nuclear factor 4 alpha (HNFα) (39). Thus, we show evidence supporting that CAR acts a tumour suppressor in the pathogenesis of liver cancer, although more thorough studies are needed.

Through our experimental findings we propose for the first time that CAR regulates the stemness of liver cancer cells and ultimately decreases the biological functioning of LCSCs (Manuscript by Bae SDW et al currently under review in *Pharmacol Res*). This study brings light to a role that CAR has not been previously implicated in, the regulation of stemness and the functioning of LCSCs. According to the current understandings of the role of LCSCs in liver cancer, decreased stemness of cancer cells will impede cancer growth and progression (41). The novelty of this study also lies within the study design whereby the patterns of expression and associations with cancer growth and progression were examined with bioinformatic analyses. Then those correlations were further explored with the utilisation of three HCC cell lines with three different ways to activate and/or modulate levels of CAR for a wide range of functional assays. We believe that this study will highlight the novelty of studying CAR as a tumour suppressor for liver cancer and show the unveiled potential of CAR as a potential target for therapy.

Based on our data, we propose that CD24 and CD133 are the main stem cell markers that drive the functional aspect of HCC growth and progression such as proliferation, migration, and invasion. CD24 is a cell adhesion molecule (48) and a widely established oncogenic molecule as well as a marker for LCSCs (49-51). CD24 is implicated in a wide range of pathways such as early growth response 1 (EGR1) (50), signal transducer and activator of transcription 3 (STAT3) (49) and protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signalling pathways (52). CAR activation decreased CD24 expression and proliferation, migration, and invasion of HCC cells. These results imply that CAR decreased the expression of CD24, and this may lead to reduced CD24 signalling and resultant decrease in proliferation, migration, and invasion of HCC cells. Up to date, CAR has been associated with cancer stem cells only in a brain cancer background whereby CAR acted as a tumour suppressor via regulating $CD133^{+}$ brain tumour stem (15). CD133 is also known to contribute greatly to liver cancer

progression and is involved in many signalling pathways but significantly in the aberrantly activated Wnt/β-catenin pathway (44). Similarly, the expression of CD133 as well as cell proliferation, migration, and invasion were reduced upon CAR activation. Activation of CAR also impeded tumour sphere forming ability of HCC cells. These results collectively point CAR to a tumour suppressor in liver cancer. An extensive literature search revealed no studies regarding the tumour suppressive role of CAR with a special focus on LCSCs. Hence, for the first time identified that CAR plays a tumour suppressive role in human liver cancer via inhibiting the activity and functions of liver cancer stem cells. Further studies are warranted to examine the underlying mechanism of action behind the tumour suppressive role of CAR in human liver cancer using more clinically relevant models such as human tissues-derived organoids and humanized *in vivo* liver cancer models.

4.2 Future directions

While our experimental findings have shown a great potential of CAR, further studies are essential to elucidate the role of CAR as liver cancer tumour suppressor. These immediate studies will include (***1***) detailed understanding of the mechanism of action of CAR in regulating the stemness of liver cancer cells and LCSCs; (***2***) investigating the possibility of targeting CAR by using the human CAR specific agonist CITCO to treat liver cancer, either alone or in combination with existing therapies; and (***3***) investigating the role of CAR in other pre-cancerous liver diseases such as MAFLD and related hepatocarcinogenesis (16), using more translational pre-clinical models such as patient-tissues derived organoids.

4.3 Concluding remarks

Overall, this project has given us a new perspective of the role of CAR in human liver cancer. We have shown that CAR regulates stemness of liver cancer cells to impeded cancer cell proliferation, migration, invasion, and tumour sphere forming ability. It has presented a novel

role of CAR in human liver cancer that still needs to be further investigated. A deeper understanding of the underlying mechanism of action will be crucial in our understanding of HCC as well as in the development of novel therapeutics and strategies against this malignancy.

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