ELUCIDATION of SORAFENIB RESISTANCE MECHANISMS IN HEPATOCELLULAR CARCINOMA

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF ENGINEERING AND SCIENCE
OF BILKENT UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
IN
MOLECULAR BIOLOGY AND GENETICS

By
Zeynep Boyacıoğlu
July, 2018

ELUCIDATION of SORAFENIB RESISTANCE MECHANISMS IN HEPATOCELLULAR CARCINOMA

By Zeynep Boyacıoğlu

July, 2018

We certify that we have read this dissertation and that in our opinion it is fully adequate in scope and quality, as a thesis for the degree of Master of Science.

_____

Serkan İsmail Göktuna

(Advisor)

_____

Fatima Susanna Faustina Aerts Kaya

_____

Murat Alper Cevher

Approved for Graduate School of Engineering and Science

_____

Ezhan Karaşan

Director of the Graduate School of Engineering and Science

Abstract:

Elucidation of Sorafenib Resistance Mechanisms in Hepatocellular Carcinoma

Zeynep Boyacıoğlu
MSc in Molecular Biology and Genetics
Advisor: Serkan İsmail Göktuna
July, 2018

Hepatocellular Carcinoma (HCC) is the sixth most common type of cancer and the second leading cause of cancer-related deaths worldwide. Currently, Sorafenib is the only approved first-line treatment option for unresectable advanced HCC patients. Although Sorafenib can be beneficial for some patients, overall survival can only be extended for 4 months. One of the main reasons is the development of Sorafenib resistance. Many studies have been conducted to decipher the mechanisms underlying this drug resistance, yet much more still awaits for elucidation. This study suggests a novel involvement of a player, an antiviral kinase, which was previously described as an oncogenic protein. Strikingly in our investigation, Sorafenib resistance seems to be caused by the loss of this interferon related kinase. This protein expression is ablated in Sorafenib resistant Hep3B cell line. Loss of this protein by shRNA confers Sorafenib resistance to naïve Hep3B cells. Reversely, protein rescue can re-sensitize these resistant cells to Sorafenib. We also report in this work for the first time that this Sorafenib resistant Hep3B cell line exhibits partial EMT signature. Over all, this study paves way for further studies investigating the implication of this antiviral protein in Sorafenib resistance mechanism in HCC.

Keywords: HCC, Sorafenib resistance, Hep3B, oncogenic antiviral proteins

Özet:

Hepatoselüler Karsinomda Sorafenib Direnç Mekanizmalarının Belirlenmesi

Zeynep Boyacıoğlu

Moleküler Biyoloji ve Genetik, Yüksek Lisans

Tez Danışmanı: Serkan İsmail Göktuna

Temmuz, 2018

Hepatosellüler Karsinom (HSK) en yaygın altıncı kanser türüdür. Ayrıca, HSK bütün dünyada kanser kökenli ölümler arasında ikinci sırada yer almaktadır. Ameliyat edilemez ileri derece HSK hastaları için onaylanmış tek tedavi seçeneği Sorafenib'dir. Ne var ki, hastalara yarar sağlamasına rağmen Sorafenib yaşam beklentisini yalnızca 4 ay uzatabilmektedir. Bunun başlıca nedenlerinden biri de direnç gelişmesidir. Sorafenib ilaç direncini kırmaya yönelik birçok çalışma olsa açıklanması gereken çok fazladır. Bu çalışmada daha önce bir onkogen olduğu gösterilen bir antiviral kinazın ilişkisi gösterilmiştir. Şaşırtıcı olarak, bizim çalışmamız bu interferon-alakalı kinaz'ın azalmasının Sorafenib direnci ile ilgili olduğunu göstermiştir. Protein ifadesi Sorafenib'e dirençli hücrelerde azalmıştır. Buna ek olarak, saf Hep3B hücrelerinden shRNA yardımı ile bu gen silindiğinde bu hücreler ilaca direnç göstermiştir. Ayrıca, dirençli hücrelerde antiviral kinaz kurtarıldığında ilaca duyarlı hale geldikleri görülmüştür. Ayrıca, bu çalışmada ilk defa Sorafenib'e dirençli hücrelerde kısmi EMT olduğu gösterilmiştir. Sonuç olarak, bu çalışma bu antiviral protein'in Sorafenib direncine etkisini incelemek için yol açacaktır.

Anahtar kelimeler: Hepatoselüler karsinom, sorafenib direnci, Hep3B, onkogenik antiviral proteinler

Acknowledgements:

I would like to thank my advisor Assist. Prof. Serkan İsmail Göktuna for giving me the opportunity to work in his lab, for believing in me and for always sharing his invaluable experiences with me throughout my Master's study.

I would like to thank deeply Dr. Tieu Lan Chau for the endless support that she gave in the past two years. She has taught me for much, both including experimental procedures and the field of science itself and I cannot thank her enough.

I would like to thank all the past and present members of Göktuna Lab for their support and help anytime I needed but most importantly for their friendship, dear İlayda Baydemir, Elif Tuğçe Karasu, Erta Xhafa, Uğur Kahya.

I would like to thank my family and friends who have been so patient with me during my Master's study; for being so understanding of why I was always late to everywhere, because I was in the lab, and for giving me more support than I could even ask for.

Lastly, I would like to thank Scientific and Technological Research Council of Turkey (TÜBİTAK) for funding my Master's studies. This thesis was supported by the grant number 116Z349.

Teşekkürler:

Tez danışmanım Sayın Yar. Doç. Serkan İsmail Göktuna'ya bana laboratuvarında çalışma fırsatını verdiği, bana her zaman inandığı ve yüksek lisans eğitimim boyunca paha biçilemez tecrübelerini benimle paylaştığı için teşekkür ederim.

Dr. Tieu Lan Chau'ya bu iki yıldaki sonsuz desteği çok teşekkür ederim. Bana hem deney teknikleri hem de bilim dünyası dahil birçok şey öğrettiği için ona ne kadar teşekkür etsem azdır.

Göktuna Lab'ın geçmiş ve şimdiki bütün üyelerine destekleri, yardımları ve özellikle arkadaşlıkları için çok teşekkür ederim, sevgili İlayda Baydemir, Elif Tuğçe Karasu, Erta Xhafa, Uğur Kahya.

Ailem ve arkadaşlarıma yüksek lisans eğitimim boyunca bana karşı sabırlı oldukları, her yere geç kaldığımda anlayışları ve tahmin edebileceğimden çok fazla destekledikleri için teşekkür ederim.

Son olarak da Türkiye Bilimsel ve Teknolojik Araştırma Kurumu'na (TÜBİTAK) yüksek lisans eğitimimi destekledikleri için teşekkür ederim. Bu tez 116Z349 numaralı TÜBİTAK projesiyle desteklenmiştir.

Contents:

List of Figures:

List of Tables:

1 INTRODUCTION:

1.1 HEPATOCELLULAR CARCINOMA (HCC):

1.1.1 Incidence and cause of HCC

Cancer is a disease that affects the majority of the world's population. Among all the cancers, liver cancer is the sixth most common cancer type and the second leading cause of cancer-related deaths (Figure 1.1 and Figure 1.2) [1,2].

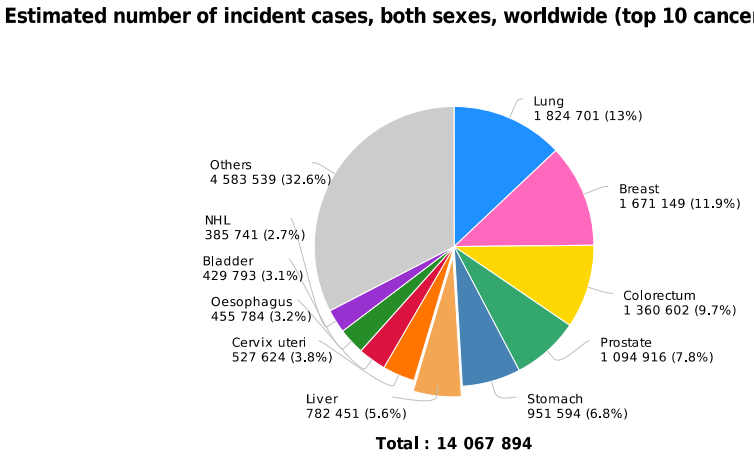


Figure 1.1: Estimated cancer cases worldwide, divided according to the cancer type. Graph taken from Ref 2.

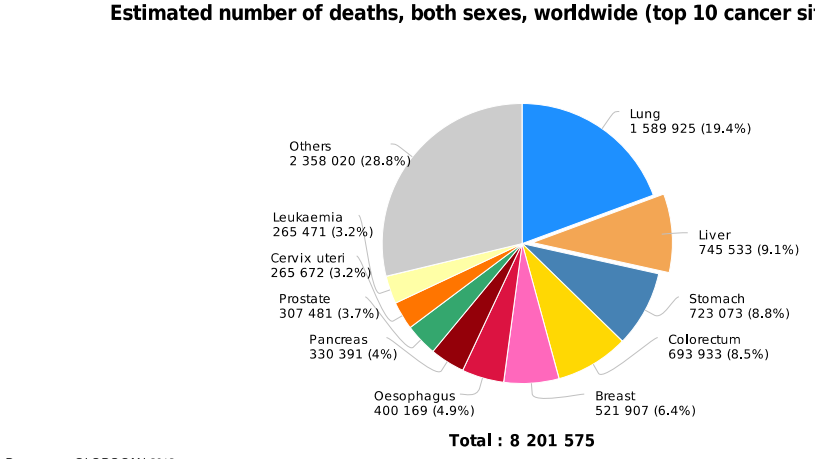


Figure 1.2: Estimated number of cancer-related deaths, divided according to cancer. Graph taken from Ref 2.

Hepatocellular Carcinoma (HCC) constitutes almost 90% of all primary liver malignancies. Main risk factors of HCC are: Hepatitis Virus B (HBV) and Hepatitis virus C (HCV) infections, cirrhosis, excessive alcohol use, Non-Alcoholic Fatty Liver Disease (NAFLD), metabolic syndrome, Aflatoxin B inhalation/consumption and smoking in considered [3]. In addition, diabetes and obesity are independent factors and mortality is five times higher in patients with higher body mass index than in patients with lower body mass index [4].

HCC development is a multi-step process. First, low-grade dysplastic pre-cancerous cirrhotic nodules (LGDNs) are formed and these LGDNs convert into high-grade dysplastic nodules (HGDNs). HGDNs are considered to have potential of being early stage HCC (Stages 0 and A) and develop to become intermediate or advanced HCC (Stages B and C) [3]. Underlying mechanism of HCC involves many mutations and alterations in many signaling pathways that control cell proliferation, angiogenesis, cell cycle regulation, telomere maintenance and epigenetic [3,4]. Among these, WNT-

β Catenin signaling pathway, RAS/RAF/MAPK pathway, PI3K/AKT/mTOR pathway and oxidative stress pathway are significant.

1.1.2 Current therapeutic treatments of HCC:

HCC is resistant to treatment with chemotherapy and radiotherapy [5]. Hence, liver transplantation is the best option for HCC patients since it enables complete treatment of both HCC and the underlying liver condition. Unfortunately, only a very small portion of the HCC patients is qualified for transplantation since the criteria for eligibility is very strict. Milan criteria suggest that only patients with a single tumor with diameter less than 5 cm or patients with at most 3 tumors with diameters less than 3 cm are candidates for liver transplantation [6]. More inclusive criteria have been proposed from a group in University of California, San Francisco (UCSF). According to UCSF criteria, patients with single tumor that is less than 6.5cm or patients with at most 3 nodules with less than 4.5cm and the total tumor diameter less than 8cm are eligible for liver transplantation [6]. Furthermore, for patients at very early or early stages of the disease, surgical resection of the tumor might be a treatment option. These patients can be selected upon their tumors; isolated tumors with persevered liver function [10]. Nevertheless, HCC at early stages is asymptomatic hence generally can only be diagnosed at later stages [7]. This reduces the number of patients than can benefit from liver transplantation. For these patients there are other surgical treatment options that are minimally invasive. First, Transarterial Chemoembolization (TACE) is an option for intermediate stage HCC patients. In this treatment, the blood supply to the tumor is inhibited to selectively target HCC cells. TACE can also be combined with Sorafenib. There are several studies that show that combining Sorafenib with TACE increases median survival of

patients when compared to TACE alone. [8,9]. Another treatment option is Radiofrequency Ablation (RFA), in which rapid electromagnetic pulses are used to ablate the tumor size. RFA can be a beneficial treatment option to reduce the size of the tumor and hinder the progression until the patient becomes a candidate for liver transplantation [10]. For patients who are not qualified for surgery, Percutaneous Ethanol Injection Therapy (PEIT) is the most common treatment option. This treatment relies on the cytotoxicity of ethanol [10].

Apart from surgical treatment, there are some medical treatments available for HCC patients. For example, Oncolytic Virus Treatment takes advantage of genetically engineered viruses to kill the tumors cells. This therapy option utilizes the fact that the viruses should infect the tumor cells and the patients' own immune system to eliminate the tumor cells. Last but not least, the immunotherapy is considered as a crucial treatment option in which antibodies against apoptosis inhibitors are given to the patients. [10]. The study by El-Khoueiry et al showed that the antibody against PD-1 inhibitor was effective in advanced HCC patients [11].

1.1.2.1 Systemic treatment of advanced HCC:

Sorafenib is an oral multi-kinase inhibitor that blocks proliferation and angiogenesis of tumor cells. It exerts its anti-proliferative effects by inhibiting kinases such as Ras and Raf in the Ras/Raf/MEK/ERK signaling pathway. On the other hand, it exerts its anti-angiogenetic effects by inhibiting kinases such as c-KIT, FLT-3, VEGFR, PDGFR. Sorafenib also has cytotoxic effects that are believed to play an important role as an anti-tumor agent [12,13]. There are two large-scale, double-blind, placebo-controlled, phase III clinical trials conducted to assess the efficacy and the safety of Sorafenib in patients with advanced HCC. The first trial was the Sorafenib Hepatocellular Carcinoma Assessment Protocol (SHARP) that showed that the overall survival (OS) of patients treated with Sorafenib is longer than patients treated with placebo (10.7 months and 7.9 months, respectively). In addition, Sorafenib also prolonged median time to progression (TTP) (5.5 months in Sorafenib arm compared to 2.8 months in placebo arm) and the disease control rate (DCR) was also higher in patients that received Sorafenib (43%) when compared to patients who received placebo (32%) [14]. These results were confirmed in another phase III, double-blind, placebo-controlled clinical trial that was conducted in the Asia-Pacific region (with OS of 6.5 months in Sorafenib arm and 4.2 months in placebo arm) [15]. The drug-related adverse events in both of the trials were similar, with the most common ones being diarrhea, hand-foot skin reaction, fatigue and anorexia [14,15]. Although there is a big difference in the OS of two different trials that might be explained due the different geographical origins of the patients; in the SHARP trial, the patients are from Western origins and the patients from the AP trial are from Asian origin. This brings upon different etiologies of HCC in different regions. For instance, the incidence of HBV infection was higher in the Asia-Pacific trial than in the SHARP

trial (73% and 12% respectively). On the other hand, 30% of the patients in SHARP trial had HCV infection while only 8.4% of the patients enrolled in Asia-Pacific trial were infected with HCV [14,15]. It is shown that patients with HBV infection have worse prognosis than patients with HCV infection [16]. Upon these findings, Sorafenib has been approved by the United States Food and Drug Administration (FDA) in 2010 as the first-line treatment option for patients with advanced HCC unresectable tumors.

Although Sorafenib is the only drug that is approved for the treatment of advanced HCC, it can only increase overall survival by up to 4 months. Thus, other drugs have been investigated to replace Sorafenib (such as Sunitinib, Brivanib, Linifenib and Lenvatinib). However, none of these candidates have proven to the superior over Sorafenib [17].

1.1.2.2 Sorafenib resistance:

Resistance against chemotherapeutic agents is commonly seen in cancer. Drug resistance can be divided into two: intrinsic resistance and acquired resistance. Intrinsic resistance is when a patient does not respond to the drug hence they can never have any benefit from drug use. On the other hand, acquired resistance happens when a patient stops responding to a drug after a period of benefit [13].

As mentioned above, Sorafenib can only increase the OS of patients by 4 months and one primary reason for this the development of resistance. There are several proposed mechanisms of Sorafenib resistance. First one is the PI3K/AKT pathway. This

pathway plays a role in cell apoptosis and over-phosphorylation of AKT is seen in resistance against Sorafenib. Inhibition of AKT results in resensitization of HCC cells against Sorafenib [12, 18]. Second, autophagy is a cell's self-protection mechanism that enables cells to survive in unfavorable conditions. However, this may promote tumor growth since it induces cell survival [19].

Furthermore, Sorafenib is an anti-angiogenic drug. These drugs might change the tumor microenvironment; causing the blood vessels to contract and blood flow to the tumor is decreased. However, this causes the selection of colonies in the solid tumor that can survive under hypoxic conditions and these cells are generally more resistant to chemotherapy [19]. Last but not least, Epithelial To Mesenchymal Transition (EMT) also plays a role in Sorafenib resistance. Two recent studies by Fischer et al and Zheng at al showed that targeting EMT resulted in reduced survival of cancer cells and reduced tumor sizes [20-22].

1.1.2.3 Treatment after Sorafenib resistance:

As mentioned earlier, patients develop resistance against Sorafenib quickly and hence a second-line treatment to be used after progression on Sorafenib was in urgent need. To this extend, several drugs have been tested in several clinical trials but unfortunately were not successful. For example, Brivanib, Everolimus, Ramucirumab and Tivatinib were among the agents investigated as treatment after progression on Sorafenib [16]. Only one multi-kinase inhibitor, Regorafenib, could show superiority to placebo in clinical trials. In the RESOURCE Trial, Regorafenib lead to an increase in OS from 7.8 months to 10.6 months, independent of the final dose of Sorafenib

[16,23]. In addition, Nivolumab, a monoclonal antibody against Programmed death-1 (PD-1), also received a fast FDA approval after showing promising results in phase I/II CheckMate 040 clinical trial [23].

1.2 EPITHELIAL-MESENCHYMAL TRANSITION (EMT):

Epithelial-to-mesenchymal transition (EMT) is a reversible multi-step progress in which the cell-cell adhesion molecules are lost and the cells gain a more migratory phenotype [24]. Furthermore, the cell polarity is lost and the cells are de-differentiated from epithelial to mesenchymal. EMT is a process that is seen during embryonic development, wound healing and tumor metastasis [25]. There are several signaling pathways that play critical roles in EMT and those pathways are generally also shown to be a part of carcinogenesis [26]. First of all, TGF-β signaling is one of the most important inducers EMT. Although TGF-β suppresses proliferation in cells, once this early effect is overcome, TGF-β acts as an inducer of EMT and contributes to cancer cell migration. Clinically, higher expression of TGF-β late response genes is associated with more metastatic tumors [27].

Snail is a transcription factor that downregulates the expression of epithelial markers, especially E-Cadherin [28]. Snail expression is induced by the Notch signaling pathway [27]. In addition, NF-κB signaling can also induce Snail and cause EMT through TNFα [28]. Hence, TNFα can be considered as another important inducer of EMT.

1.2.1 EMT and drug resistance:

Other than being involved in metastasis, EMT is recently shown to play a role in chemoresistance [20, 21, 29]. Zhang et al. showed that EMT is regulated by several other pathways such as PI3K/AKT, MAPK and Ras/ERK pathways that are required for the maintenance of the mesenchymal state [29]. As mentioned above, upregulation of the PI3K/AKT pathway is associated with Sorafenib resistance. Zhang et al. proved that hyperactive PI3K/AKT signaling pathway is responsible for EMT in HCC [29].

1.2.2 Partial EMT:

EMT is seen in many aspects of embryogenesis, as well as wound healing and tumor progression. However, the EMT process is not always complete during metastasis. In fact, cells that have both epithelial and mesenchymal properties can be observed. This dual property confers them to a more advantages while leaving the bloodstream [42]. In addition, there are studies that show that the tumors that possess both epithelial and mesenchymal properties are more invasive. Recently, Yamashita et al showed that the breast cancer samples that express both E-Cadherin and Vimentin are also more aggressive [41].

1.3 IKBKE GENE:

Inhibitor of Nuclear Factor Kappa-b kinase epsilon (IKKε) protein is expressed from the IKBKE gene in the q arm of Chromosome 1 [30]. It has been shown to play important roles in inflammation, cell survival, cell proliferation and cell growth through the IRF3/7 and NF-κB pathways [31,32]. In addition, IKBKE is also shown to have roles in carcinogenesis in many malignancies. Not only IKBKE is overexpressed in some cancers, high expression of IKBKE is also associated with drug resistance [32]. Last but not least, IKBKE induces cell proliferation and cell growth [32].

The IKBKE gene has been shown to act as an oncogene in many cancers such as breast cancer, glioma, prostate cancer and ovarian cancer. In addition, IKBKE is also associated with cisplatin resistance in ovarian cancer and tamoxifen resistance [32, 38].

Upon inflammation, IKBKE is activated and IKBKE subsequently activates IRF3/7 and STAT1 [32]. In addition, upon TLR3 activation (by ligand such as dsRNA), the IKBKE activates both IRF-3 and NF-κB, which results in the induction of IFN-β [45].

1.3.1 NF-κB signaling pathway:

NF-κB is a transcription factor that regulates many genes involved in inflammation, immunity, cell proliferation, differentiation, survival, DNA damage and cell cycle regulation [33]. As NF-κB is involved in cell survival and targets anti-apoptotic genes, it should be tightly regulated. In unstimulated cells, NF-κB is found bound to

Inhibitor of κB (IκB), which blocks its nuclear localization signal and sequesters NF-κB in the cytosol [34]. Upon induction, IKBKE phosphorylates IκB, causing its degradation and subsequent release and nuclear translocation of NF-kB. This leads to the expression of target genes regulating many cellular processes like cell survival (Figure 1.3).

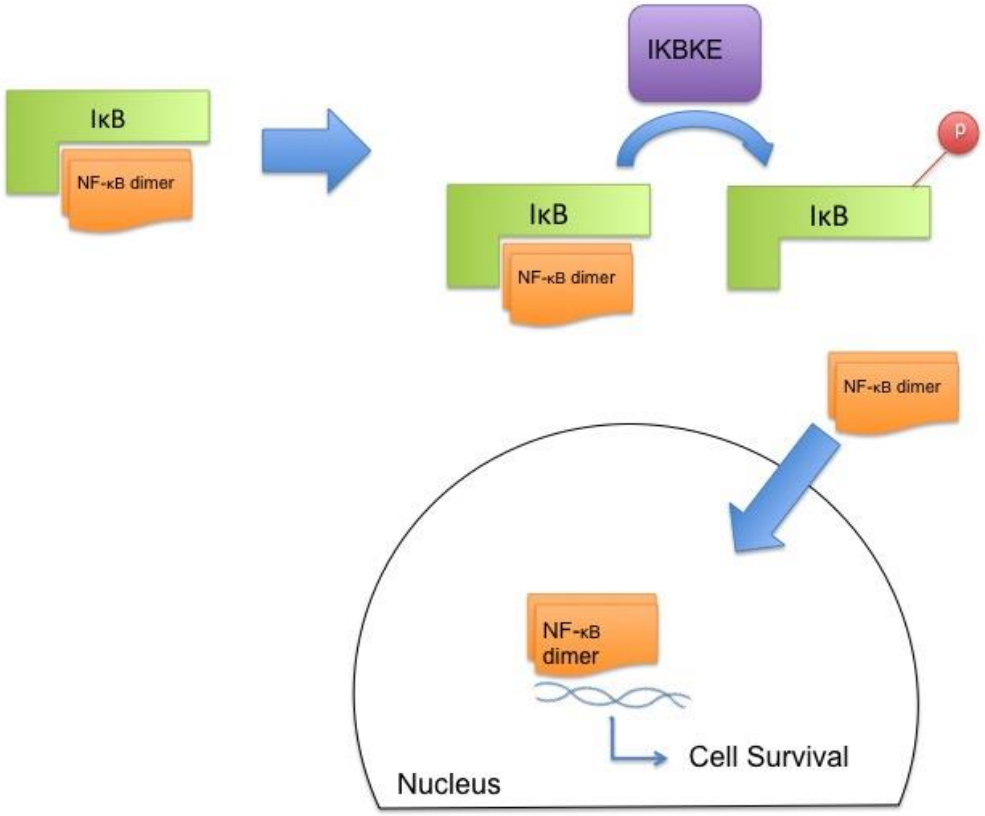


Figure 1.3: NF-κB pathway. Pathway adapted from Ref 33.

1.4 Aim of the study:

This study aims to understand the resistance mechanisms of Sorafenib in HCC cells. To this extend, naïve Hep3B cells were continuously treated with Sorafenib for 8 months to generate Sorafenib-resistant SorRHep3B cells. These cells were characterized and IKBKE-dependent resistant mechanism was investigated. In addition, the effects of IKBKE depletion and inhibition on Sorafenib response were also explored.

2 Materials and Methods:

2.1 Materials:

2.1.1 Chemicals and reagents:

Product Name:	Catalog No:	Company, Country
PageRuler Prestained Protein L	SG-2661	Themo Fisher Scientific, USA
40% Acrylamide/Bis Solution, 37.5:1	1610148	Bio-Rad,USA
Hiperfect Transfection Reagent	301705	Qiagen, Germany
GIPZ LentiviralAL shRNA transduction starter kit IKBKE	RHS5086	Dharmacon/GE Healthcare, UK
cOmplete Protease Inhibitor Cocktail	11697498001	Roche, USA
Bovine Serum Albumin (BSA)	sc-2323	Santa Cruz Biotechnology, USA
Glycine	GLN001.1	Bioshop Canada, Canada
polyFect	1015586	Qiagen, Germany
Ammonyum persulphate	A2941	Applichem, USA
Calcium chloride	C3306	Sigma Aldrich, USA
EDTA	E-5134	Sigma Aldrich, USA
Ethanol	32221	Sigma Aldrich, USA
Glycerol	15524	Sigma Aldrich, USA
Hydrochloric acid	100317	Merck, USA
2-Propanol	100995	Merck, USA
2- Mercaptoethanol	805740	Merck, USA
di Sodyum hydrogen phosphate dihydrate	1.06580.	Merck, USA
Sodyum dodecyl sulfate	822050	Merck, USA
Tween	777	Ambresco/VWR, USA
Tris hydrochloride	234	Ambresco, USA
Trizma Base	T1503	Sigma Aldrich, USA

2.1.2 Cell culture media:

Product Name:	Catalog No:	Company, Country
DMEM	BE12-707F	Lonza, Switzerland
DMEM	31885023	Gibco, USA
optiMEM	31985070	Gibco, USA
Pensicin/Streptomycin	DE17-602E	Lonza, Switzerland
L-Glutamine	BE17-605E	Lonza, Switzerland
DPBS	BE17-512F	Lonza, Switzerland
Trypsin-EDTA	BE17-161E	Lonza, Switzerland
Trypsin-EDTA	25200056	Gibco, USA
Fetal Bovine Serum (FBS)	S181H-500	Biowest, France
Polybrene (10mg/ml)	sc-134220	Santa Cruz, USA

Geneticin (G418)	10131-027	Gibco, USA
Puromycin	ant-pr-1	Invitrogen, USA
Sorafenib	S7397-200MG	Selleckchem, USA
Amlexanox	A2401	Tokyo Chemical Industry, Japan
DMSO	67-68-5	Applichem, USA

2.1.3 Kits:

Product Name:	Catalog No:	Company, Country
iScript cDNA Synthesis Kit	1708891	Bio-Rad, USA
SYBR® Premix Ex Taq™ II (Tli RNase H Plus)	RR820W	Takara Bio, Japan
Pierce BCA Protein Assay kit	LSG-23227	Thermo Fisher Scientific, USA
Pierce ECL western blotting substrate	LSG-32106	Thermo Fisher Scientific, USA
SuperSignal West Femto Maximum Sensitivity Substrate	34094	Thermo Fisher Scientific, USA
CellTiter-Glo® Luminescent Cell Viability Assay	G7570	Promega,USA
Caspase-Glo® 3/7 Assay	G8091	Promega,USA
E.Z.N.A. Total RNA Kit I	R6834-02	Omega Bio-Tek, USA

2.1.4 Consumables:

Product Name:	Catalog No:	Company, Country
5mL serological pipets	4487	Corning, USA
10mL serological pipets	4488	Corning, USA
25mL serological pipets	4489	Corning, USA
1000uL filter tips	740288	Greiner-BİoOne, Germany
200uL filter tips	739288	Greiner-BİoOne, Germany
20uL filter tips	774288	Greiner-BİoOne, Germany
10uL filter tips	F161630	Greiner-BİoOne, Germany
Loading tips	14-222-809	Axygen, USA
1000uL tips	551146	LP Italiana SP, Italy
200uL tips	4845	Corning, USA
10uL tips	F161630	Gilson, USA
1.5mL SafeLock Tubes	30120086	Eppendorf, Germany
1.5mL Reaction Tubes	3621	Corning, USA
2 mL Reaction Tubes	430659	Corning, USA

15mL Falcons	62.554.502-500	Sarstedt, Germany
50mL Falcon	62.547.254-300	Sarstedt, Germany
Non-sterile 96-well plates	3655101	Greiner-BİoOne, Germany
LightCycler 480 Multiwell 96	472969201	Roche, Switzerland
RTCA CIM plates	566581701	Acea BioSciences, USA
RTCA E-Plate	546983001	Acea BioSciences, USA
96-well plates	655180	Greiner-BİoOne, Germany
12-well plates	665180	Greiner-BİoOne, Germany
6-well plates	657160	Greiner-BİoOne, Germany
145mm tissue culture dish	639160	Greiner-BİoOne, Germany
100mm tissue culture dish	664160	Greiner-BİoOne, Germany
60mm tissue culture dish	628160	Greiner-BİoOne, Germany
35mm tissue culture dish	627160	Greiner-BİoOne, Germany
175cm tissue cultre flask	660175	Corning, USA
75cm tissue culture flask	658175	Corning, USA
25cm tissue culture flask	690175	Corning, USA
Cryovials	121263	Greiner-BİoOne, Germany
Immobilion-P PVDF Membrane	1620177	Millipore, USA
Whatman Paper	732-4093	Whatman, UK

2.1.5 Buffers:

Buffer:	**Composition:**
10X Running Buffer	144g Glycine, 10 gr SDS, 30.2g Tris-Base up to 1L water
10X Transfer Buffer	144g Glycine, 30.2g Tris-Base up to 1L water
10X TBS-T	24.2g Tris-Base, 80g NaCl set pH=7.6 20mL Tween, up to 1L water

Stacking Gel	0.6mL Bis-Acrylamide, 3.5mL water 625uL 1M Tris pH=6.8, 125uL 0.25M EDTA, 100uL 10%SDS 60uL 1*% APS, 10uL TEMED
8% Separating Gel	3.2mL Bis-Acrylamide, 5.8mL water, 1.5mL Glycerol, 3.75mL 1.5M Tris pH=8.8 375uL 0.25M EDTA, 300uL 10% SDS 125uL 10% APS, 15uL TEMED
2X HBSS	10mL 1M Hepes, 666uL 3M KCl, 0.4g Dextrose, 11.2mL 5M NaCl, 0.0531g Na2HPO4, up to 200mL water ph=7.01
50X TAE Buufer	242g Tris-Base, 57.1mL Acetic Acid 100mL 0.5M EDTA, up to 1L water
2X Cell Lysis Buffer Stock	25mL 1M Hepes, 30mL 5M NaCl, 5mL Triton-X-100, 100mL Glycerol 340mL water
1X Cell Lysis Buffer	5mL 2X cell lysis buffer stock, 500uL Na3VO4, 500uL NaF, 500uL b-Glycerophosphate, 500uL cOmplete protease inhibitor up tp 10mL water
1X PBS	1.702g Na2HPO4, 8g NaCl, 0.2g KCl, 0.2g KH2PO4, up to 1L water
Mild Stripping Buffer	3g Glycine, 0.2g SDS, 20mL Tween, up to 200mL water, pH= 2.2

2.1.6 List of Antibodies:

Product Name:	Catalog No:	Company, Country
(human) IKKε D20G4	2905S	Cell Signaling Technoloy, USA
pIKKε (S172) D1B7	8766S	Cell Signaling Technoloy, USA
TBK1/NAK	3031S	Cell Signaling Technoloy, USA
pTBK1 (S172) D52C2	5483S	Cell Signaling Technoloy, USA
pERK1/2 Thr202 pP44/42 Tyr204	4370S	Cell Signaling Technoloy, USA
pAKT S473 D9E	4060S	Cell Signaling Technoloy, USA
Vimentin D21H3	5741S	Cell Signaling Technoloy, USA
ZO-1 (D6L1E)	13663S	Cell Signaling Technoloy, USA
N-cadherin 13A9	14215S	Cell Signaling Technoloy, USA
α-tubulin	T5168	Sigma-Aldrich,USA
Cleaved Caspase 3 (Asp175) 5A1E	9664S	Cell Signaling Technoloy, USA
ERK 1 Antibody (G-8)	sc-271269	Santa Cruz Biotechnology, USA
IKK-i Antibody (A-11)	sc-376114	Santa Cruz Biotechnology, USA
E-cadherin Antibody (G-10)	sc-8426	Santa Cruz Biotechnology, USA
TANK Antibody (D-2)	sc-166643	Santa Cruz Biotechnology, USA
Twist Antibody (Twist2C1a)	sc-81417	Santa Cruz Biotechnology, USA
αMouse	7076S	Cell Signaling Technoloy, USA
αRabbit	7074S	Cell Signaling Technoloy, USA

2.1.7 Sequences of primers:

Primer Name	SEQUENCE (5' --> 3')
h_IKBKE _Fw	TGCGTGCAGAAGTATCAAGC
h_IKBKE Rev	TACAGGCAGCCACAGAACAG
h_TBK1 _Fw	GTGGTGGGTGGAATGAATCAT
h_TBK1 Rev	ATCACGGTGCACTATACCATTCTC
h_CDH1_Fw	CCCGGGACAACGTTTATTAC
h_CDH1_Rev	GCTGGCTCAAGTCAAAGTCC
h_KRT18_Fw	TGATGACACCAATATCACACGA
h_KRT18_Rev	GGCTTGTAGGCCTTTTACTTCC
h_ZO1_Fw	CAGAGCCTTCTGATCATTCCA
h_ZO1_Rev	CATCTCTACTCCGGAGACTGC
h_CDH2_Fw	ACAGTGGCCACCTACAAAGG
h_CDH2_Rev	CCGAGATGGGGTTGATAATG
h_ZEB1_Fw	GGGAGGAGCAGTGAAAGAGA

h_ZEB1_Rev	TTTCTTGCCCTTCCTTTCTG
h_ZEB2_Fw	AAGCCAGGGACAGATCAGC
h_ZEB2_Rev	CCACACTCTGTGCATTTGAACT
h_FN_Fw	CTGGCCGAAAATACATTGTAAA
h_FN_Rev	CCACAGTCGGGTCAGGAG
h_SNAI2_Fw	TGGTTGCTTCAAGGACACAT
h_SNAI2_Rev	GTTGCAGTGAGGGCAAGAA
h_MMP9_Fw	GAACCAATCTCACCGACAGG
h_MMP9_Rev	GCCACCCGAGTGTAACCATA
h_GAPDH_Fw	GCCCAATACGACCAAATCC
h_GAPDH_Rev	AGCCACATCGCTCAGACAC
h_EPCAM _Fw	CGCAGCTCAGGAAGAATGTG
h_EPCAM_Rev	TGAAGTACACTGGCATTGACG
h_Claudin7 _Fw	CCACTCGAGCCCTAATGGTG
h_Claudin7_Rev	GGTACCCAGCCTTGCTCTCA
h_KRT19 _Fw	CTTCCGAACCAAGTTTGAGAC
h_KRT19_Rev	GAATCCACCTCCACACTGAC
h_Vimentin _Fw	GGTGGACCAGCTAACCAACGA
h_Vimentin_Rev	TCAAGGTCAAGACGTGCCAGA

2.1.8 IKBKE overexpression plasmids:

The IKBKE overexpression plasmids were kindly provided by Dr Alain Chariot.

2.2 METHODS:

2.2.1 CELL CULTURE:

2.2.1.1 Cell line and the maintenance:

Hep3B cells were maintained in DMEM growth medium supplemented with 5% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin and 1% L-Glutamine. Medium was changed regularly every 2-3 days and cells were passaged when they reached 80-90% confluency with a subcultivation ratio of at least ¼, to keep them healthy.

2.2.1.2 Cryopreservation of cells:

Culture medium was discarded and cells were washed carefully with PBS. After a few minutes upon adding Trypsin/EDTA to cells, watch under the microscope when cells start to dissociate from each other, then remove Trypsin before cells detach from the dish. Fresh medium was added to collect cells, followed by 5 minutes centrifugation at 1500rpm. Supernatant was discarded and the cell pellet was resuspended in growth medium supplied with 5% DMSO. Cryovials of cells were placed into the Mr. Frosty Cell Freezing Container and put directly to -80^{o}C. Cells were kept at -80^{o}C for short-term use or at N_2 liquid for long-term storage.

2.2.1.3 Thawing cells:

Cryovial of cells was thawed in the water bath until there is an ice ball remaining inside. Fresh growth medium was added in the vial drop-by-drop to reduce shock to cells due to temperature change. Next, cells were collected into a 15mL falcon and centrifuged at 1000 rpm for 3-5 minutes. Supernatant was discarded; cell pellet was resuspended in fresh growth medium and transferred into a clean cell culture dish. Gently shake the dish up-down-around for equal distribution and placed it at 37°C 5%CO_2 in the cell incubator.

2.2.1.4 Enzyme-based Mycoplasm test:

1mL supernatant was taken from the cell medium. Cell debris was removed by spinning at 13000 rpm for 5 minutes. 100µL of the clear medium was placed into 1 well of an opaque 96 well plate, added with 100µL reactant from the "Luminescence Mycoplasm Kit". Following an incubation at room temperature for 5 minute, the luminescence was read and recorded as "Read A". 100µL Substrate from the same kit was added into the well, incubated at room temperature for 10 minutes. Luminescence was recorded as "Read B".

$$\frac{Read\ A}{Read\ B}\begin{cases} < 0.9 \rightarrow no\ mycoplasm \\ = 0.9 - 1.1 \rightarrow measure\ again\ 2 - 4\ hours\ later \\ > 1.2 \rightarrow mycoplasm\ positive \end{cases}$$

2.2.1.5 PCR-based mycoplasma test:

100µL medium was taken from a confluent cell plate, then boiled for 5 minutes at 95ºC. PCR reaction mixture was prepared as follows for each condition: 24.5µL Rehydration Buffer (supplied in the kit) + 0.5µL JumpStart Taq Polymerase enzyme. For positive control, 25µL of the reaction mixture was used; for negative control: 23µL reaction mixture + 2µL nuclease free water; for samples to be checked: 23µL reaction buffer + 2µL boiled medium. After sitting at room temperature for 5 minutes, reaction tubes were placed in thermal cycler for Polymerase Chain Reaction with the program shown in Table 1.

Table 1: the conditions for PCR for mycoplasm testing.

Temperature (ºC)	Time	Number of cycles
94	2 minutes	1
94	30 seconds	40
55	30 seconds	
72	40 seconds	
4	∞	

The results were evaluated by running the reaction on 1.5% Agarose gel (with TAE Buffer) at 100V.

2.2.1.6 Development of SorRHep3B cells:

First, the IC_{50} of Sorafenib of naïve Hep3B cells was determined using Cell Titer Glo assay (described below). Next, Sorafenib resistant Hep3B cells were generated by continuous exposure to gradually increasing concentrations of Sorafenib. Sorafenib concentration was started just below the IC50 (2μM), then increased by either 0.25μM or 0.5μM every three passages. The cells are considered to be resistant after their IC_{50} is increased sufficiently. The cells were cultured in normal medium supplemented with Sorafenib. To perform experiments, resistant cells were first passaged without Sorafenib and maintained in drug-free medium no more than 7 days.

2.2.1.7 Sorafenib IC_{50} determination:

IC_{50} values for different cells lines against Sorafenib was measured by seeding the cells to 96 well plates such that they would be 80% confluent on the following day (the number of the cells differs for each cell line). Cells were treated with different concentrations of Sorafenib and incubated in 37^{o}C 5% CO_2 incubator for 72 hours. At the end of the treatment, cell viability was measured with Cell Titer Glo (explained at Page 24) and the concentration of Sorafenib that kills 50% of the cells (IC_{50}) was calculated using Nonlinear regression in GraphPad Prism Program.

2.2.1.8 IKKε overexpression in cells:

To over-express IKKε in Hep3B and SorHep3B in a well of 96 well plate: 0.2μg DNA was added to 30μL Optimem in a 1.5mL SafeLock Eppendorf tube. Following a soft vortex and quick spin down, 0.4μL PolyFect was added to the mixture. The mixture was softly vortexed and quickly spinned down again and let it sit at room temperature for 15 minutes before adding to the cell medium. Transfected cells were treated with Sorafenib 24 hours later.

2.2.1.9 shRNA transfection:

HEK293T cells were cultured in DMEM supplemented with 8% FBS. The day before the transfection, HEK293T cells were seeded to 6-well plates 8-9x10^5 cells/well to achieve 80% confluency next day. Cells were transfected with 8μg of DNA in total. DNA mixture is prepared with plasmid, H_2O and $CaCl_2$ in amount listed in Table 2. The mixture is then added drop-wise to the 2x HBSS under continuous agitation by a vortex machine. The mixture was incubated at room temperature for 3 minutes then added drop wise to HEK293T cells. The dish was gently shaken to distribute the mixture well.

Table 2: The reaction conditions for transfection of shRNA against IKBKE.

	Concentration (mg/ml)	4µG DNA	Packaging plasmid (µL)	ddH_2O (µL)	$CaCl_2$ (µL)	2X HBSS (µL)
shIKBKE Scr	1.1	3.64µL	4.3	123.06	19	150
shIKBKE 29	1.1	3.64µL	4.3	123.06	19	150
shICKBKE 43	1.2	3.33µL	4.3	123.37	19	150
shIKBKE 67	1	4µL	4.3	122.7	19	150

16 hours after the transfection, medium of transfected HEK293T cells was changed to a minimum amount in order to concentrate the viral product prepared for transduction.

The recipient cells were also previously seeded so that they are 30-40% confluent on the day of transduction. 48h post transfection, supernatant harboring virus from the producing HEK293T cells were collected, centrifuged to remove cell debris at maximum speed for 10 minutes, and then added to recipient cells. Polybrene was used at a final concentration of 5µg/ml to enhance transduction efficiency. Successfully transduced cells were selected by culturing them with puromycin for at least 2 passages before checking for the depletion of protein of interest.

2.2.2 Cell based Assays:

2.2.2.1 Cell viability assessment:

Cell viability assessment was carried out in 96 well plates with the Cell Titer Glo kit (Promega). At the ending point of experiments, the medium of the wells to be measured was replaced with 50μM fresh growth medium. Cell plate was incubated again in 37^{o}C 5% CO_2 incubator for 1.5 hours to settle down any disturbance for cells. After an additional 30 minutes incubation at room temperature, the plate is ready for the measurement with Cell Titer Glo kit. 50μL of reconstituted Cell Titer Glo was added to each well and plate, followed by 5 minutes shaking on plate shaker to assist cell lysis and10 minute incubation at room temperature to stabilize the signals. Cell lysates were next transferred to a white opaque walled 96 well plate and luminescence was to be read in Synergy HT Microplate reader.

2.2.2.2 Cell proliferation assessment with Real Time Cell Analyzer:

Before seeding the cells, 75μL of medium was added to each well of an E-Plate, by reverse pipetting to avoid bubbles, then the E-Plate is inserted to the RTCA machine and read for background measurement. SorRHep3B cells and DMSO cells were seeded at 7500 cells/well (in 75μL). Hep3B-shIKBKE cells were seeded at 10000 cells/well (in 75μL), again by reverse pipetting. The plate was incubated in the

incubator for 30 minutes to let the cells attach before proliferation measurements starts. Cell index was measured every 10 minutes for 120 hours.

2.2.2.3 Sorafenib-Amlexanox combination treatment:

Naïve Hep3B cells were seeded to 96-well plates at 10000 cells/well to be 80% confluent the next day. The cells were treated with Sorafenib and Amlexanox at the same time for 72 hours with different combination of concentrations for 72 hours. Later, conditioned medium was replaced with 50µL fresh growth medium to avoid potential cross reaction of chemicals with Cell Titer Glo reagent and cell viability was assessed, as previously described.

2.2.2.4 poly(I:C) treatment:

The day before the treatment, cells were seeded to be 70-80% confluent on the next day. Next day, medium on top of the cells was replaced with fresh growth medium containing 50µg/mL poly(I:C). Cells were lysed 24 hours later.

2.2.3 Protein-based assays:

2.2.3.1 Protein extraction from cells:

The cells were taken out of the incubator and put directly on ice to halt all reactions. Medium was aspirated out and the cells were washed with cold 1x PBS twice. Cells were scraped inside PBS using cell scraper and collected into a 1.5mL Eppendorf

tube. Cell pellet was separated by centrifugation at the highest speed for 5 minutes at +4ºC. Cell pellet was resuspended in Cell lysis buffer (Formula is given in materials section). The suspension was further incubated on ice for 15 minutes for complete cell lysis. Cell debris was separated by centrifugation at the highest speed for 10 minutes at +4ºC and the lysate was collected in another Eppendorf tube and kept at -80°C for further analysis.

2.2.3.2 Proteins quantification:

Cell lysates were thawed directly on ice. Protein quantification is performed using BCA kit according to the manufacturer's instructionss. Bovine Serum Albumin (BSA) 2µg/µl is diluted 20x with dH_2O (75µL BSA 2µg/µl +1425µL dH_2O). The standards were prepared in a 96 well as vertical triplicates with different concentrations as shown in Table 3. Samples to be measured were diluted 1:100 by adding 4µL cell lysate to 396µl dH_2O and well mixed. Diluted samples were loaded horizontal triplicate to the 96well plate. BCA reagent mixture was prepared as instructed (5mL Solution A + 100µl Solution B) and 100µl BCA was added to each well of the standards and the samples. The plate was incubated at 60ºC for 30 minutes and then the signal was read in the Synergy HT microplate reader at 562nm. The concentrations of the proteins were calculated according to the standard line.

Table 3: preparation of protein quantification standards.

Concentration (µg /µl)	**Diluted BSA(µl)**	**dH_2O (µl)**
0	0	100
1	10	90
2	20	80
3	30	70
4	40	60
5	50	50
7	70	30
10	100	0

2.2.3.3 Western Blotting:

Stacking and Separating gels were prepared according to the formula given in the materials section. After the proteins were isolated and quantified, 4x loading dye was added to the proteins and the samples were boiled at 75^{o}C for 10 minutes. Equal amounts of protein were loaded to the stacking gel. The gel was run at 120V for 100 minutes inside the Western Blot 1X Running Buffer. After the loading dye reaches the end of the gel, the run was stopped and the gel was placed inside Western Blot 1x Transfer Buffer. Sponges and Whatman papers were wetted in the same Western Blot 1x Transfer Buffer. The PVDF membrane was activated by shaking for 30 seconds in 100% methanol, later the membrane was also put in Western Blot 1x Transfer Buffer.

Once every item was wet and ready to use, the transfer sandwich was prepared:

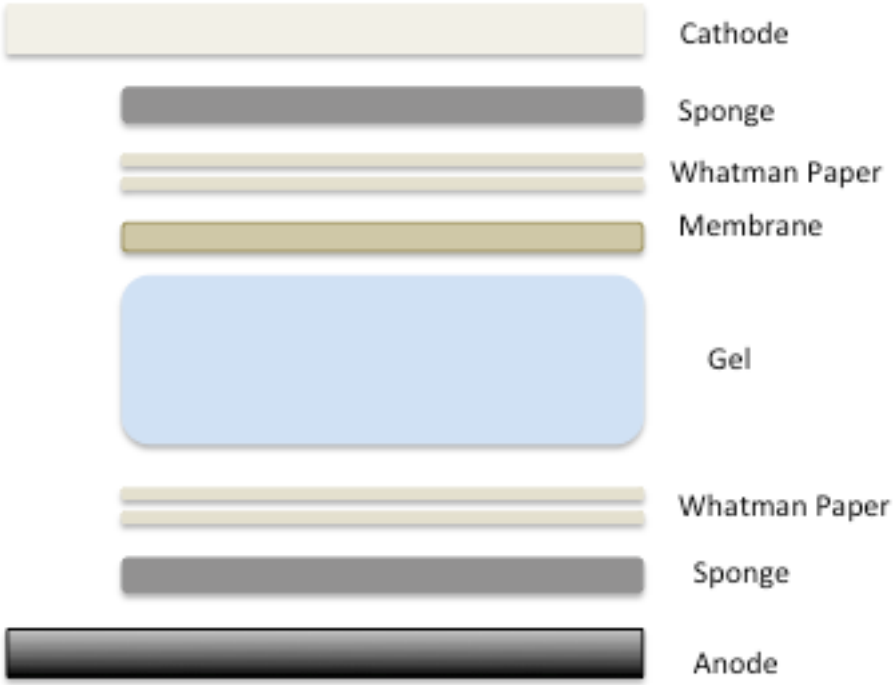


Figure 2.1: The sandwich for Western Blot transfer.

The sandwich was placed in the tank with Western Blot 1x Transfer Buffer. The proteins were transferred to the membrane for 2 hours at 250mA (or overnight at 30mA). After the transfer was done, the membrane was blocked for 1 hour in 10% milk (milk powder dissolved in 1x TBS-T) while shaking. The excess milk was washed away by shaking the membrane in 1x TBS-T 3 times 10 minutes. The membrane was cut according to the sizes of the proteins that will be checked and put in primary antibody overnight (prepared in 3% BSA in 1x TBS-T). The excess primary antibody was washed away in 1x TBS-T by shaking 3 times 10 minutes and the membrane was put to secondary antibody for 1 hour. The excess secondary antibody was washed away shaking 3 times 10 minutes in 1x TBS-T. After the washing was done, the membrane was incubated with ECL for 5 minutes and developed using Amersham Western Blot Imager.

2.2.4 RNA-based assays:

2.2.4.1 RNA isolation:

Medium was aspirated from the cells and the cells were washed with cold 1x PBS twice. 1x PBS was added on top of the cells and the cells were scraped out of the dishes using a cell scraper. The cell suspension was centrifuged for 5 minutes at maximum speed at +4ºC. Later, the supernatant was discarded and RNA was isolated from the remaining cell pellet according to the instructions supplied by the kit manufacturer. The concentration of RNA was measured by Thermo Fisher NanoDrop One.

2.2.4.2 cDNA synthesis:

The cDNA generation reaction was prepared as shown in Table 4. The enzyme should be added last to the mixture and the mixture should always be prepared on ice.

Table 4: The reaction mixture for cDNA generation

DNA (µg)	4X primer Mix (µl)	Nuclease Free Water (µl)	Reverse Transcriptase (µl)	Total (µl)
1	4	variable	1	20

After the preparation of the mixture, the tubes were quickly spinned down. The reaction was held in thermal cycler according to the conditions shown in Table 5.

Table 5: The conditions for cDNA generation.

Temperature (^oC)	Time (minutes)
25	5
46	20
95	1
4	∞

2.2.4.3 Quantitative PCR

The qPCR reaction mixtures were prepared as shown in Table 6.

Table 6: The reaction mixture of qPCR.

Samples:	cDNA stock (µl)	20	Add 3 µl from this dilution
	H_2O (µl)	440	
Primers	Primer mixture (µl)	0.5	
	SYBR Green (µl)	5	
	H_2O	1.5	
	Total	10	

Later, the plate was sealed very tightly and centrifuged for 3 minutes at 1200rpm. The reaction was held in Roche Light Cycler 480. The reaction conditions were as described in TAKARA SYBR Green instructions manual.

2.2.5 Statistical Analysis:

Unpaired t-Test was used for determining the differences between the datasets.

F-Test was used to test the differences between variance the curves. For all the analysis, GraphPad Prism is used.

3 RESULTS:

3.1 Creation of Sorafenib resistant Hep3B cell line (SorRHep3B) line

3.1.1 Determination of Sorafenib IC_{50} of naïve Hep3B cells:

In order to create the Sorafenib resistant Hep3B cell line (SorRHep3B), the naïve Hep3B cells were continuously exposed to increasing doses of Sorafenib, starting with a concentration just below the IC_{50}. Therefore, first the IC_{50} of our Hep3B cells needs to be measured. Cells seeded to a 96-well plate so that they would become 80% confluent the next day are treated with different concentrations of Sorafenib. After 72 hours, cell viability was assessed by Cell Titer Glo assay. Upon calculation with non-linear regression GraphPad Prism, the IC_{50} of the naïve Hep3B cells is obtained as 2.4μM (Figure 3.1).

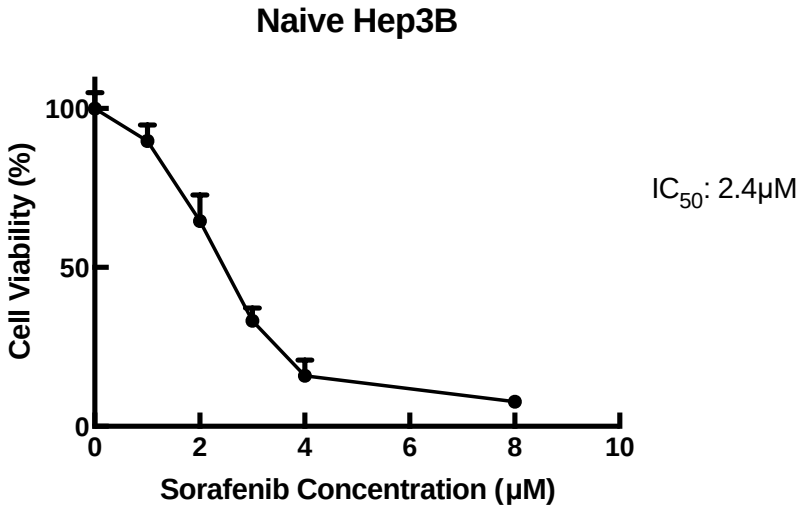


Figure3.1: IC_{50} of naïve Hep3B cell line. Naïve Hep3B cells are exposed with increased concentrations of Sorafenib for 72 hours and their viability is measured with Cell Titer Glo assay. Experiment has been performed twice with triplicates for each condition.

3.1.2 Generation of SorRHep3B cell lines:

To obtain the SorRHep3B cell line, we first start to treat naïveHep3B cells with 2μM Sorafenib and gradually increase drug concentration over time. Cells were passaged three times at the same drug concentration before being exposed to further increase by either 0.5μM or 0.25μM. Two different cell lineages were created: SorRHep3B-A and SorRHep3B-B, respectively. The schematic description is shown in Figure 3.2. Cell morphology was carefully observed and recorded. Changes in cell shape first recorded after 6 months (when the cells were growing in 2.5uM) and after 8 months we assessed the IC_{50} of these cell lines again. Both lineages showed significant increase of IC50 value: 5.758 for SorRHep3-A and 5.852 for SorRHep3B-B. We tried to expose cells further at 3.25μM or 3.5μM but cells could not handle these concentrations anymore. Hence, we decided to make investigations with cells stably growing in 3μM Sorafenib and consider them as SorHep3B, resistant to Sorafenib.

To carry out experiment with these SorHep3B, the cells were passaged without drug and kept in medium without drug for at least 4 day and at most 7 days. After 7 days, the cells were treated with Sorafenib again to reinforce the resistance.

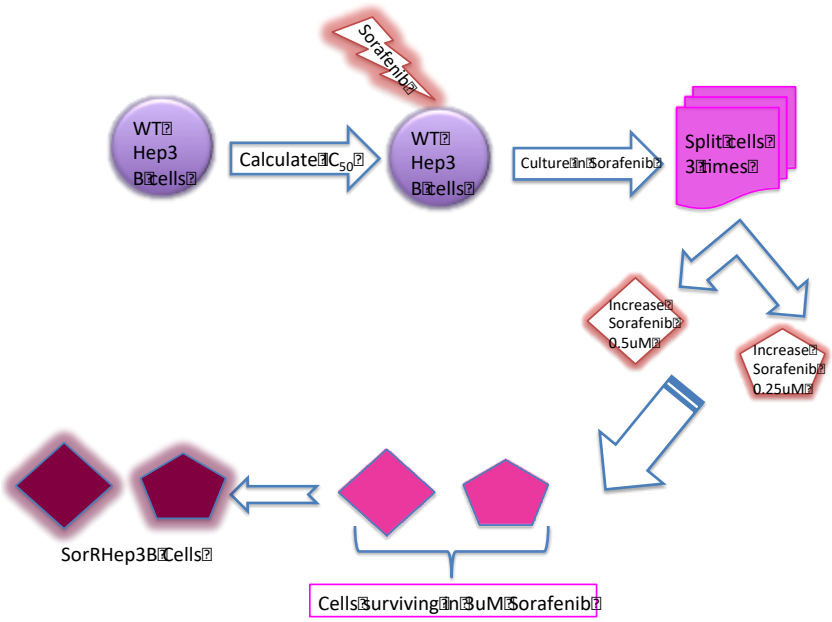


Figure 3.2: Schematic description of SorRHep3B creation. Naïve Hep3B cells were treated with gradually increasing concentrations of Sorafenib over 8 months and Sorafenib-resistant SorRHep3B cells were established.

3.1.3 Morphological differences of SorRHep3B Cells compared to Control cells:

Hep3B are epithelial, adherent cells. While DMSO-treated cells look pretty similar to the naïve cells, SorRHep3B cells showed obvious different morphological features. They are much bigger and highly branched. At high confluency, DMSO treated cells become more packed while SorRHep3B cells could never be compacted. SorRHep3B cells are healthier with big spaces among them. In addition, control cells could survive better when they were in lower confluency as compared to the SorRHep3B cells, which seem to be very fragile. Last but not least, SorRHep3B cells detached from the tissue culture dishes easier than the control cells (Figure 3.3).

Figure 3.3: SorRHep3B cells are morphologically different than DMSO cells. These images are taken with 4X magnification. Left: Hep3B cells cultured in 3µM DMSO. Middle and Right: SorRHep3B cells cultured in 3µM Sorafenib.

3.1.4 IC_{50} of SorRHep3B cell lines:

Both DMSO-treated and two lineages of SorRHep3B cells were seeded to 96-well plate at 6000 cell/well. The next day, the cells were 80% confluent and Sorafenib treatment was started. The cells were treated with different concentrations of Sorafenib for 72 hours. The cell viability was measured by Cell Titer Glo assay. The IC_{50} of SorRHep3B cells were significantly higher than that of the DMSO treated cells when compared to (5.758 and 5.82 for SorRHep3B-A and SorRHep3B-B, respectively versus 3.244, Figure 3.4).

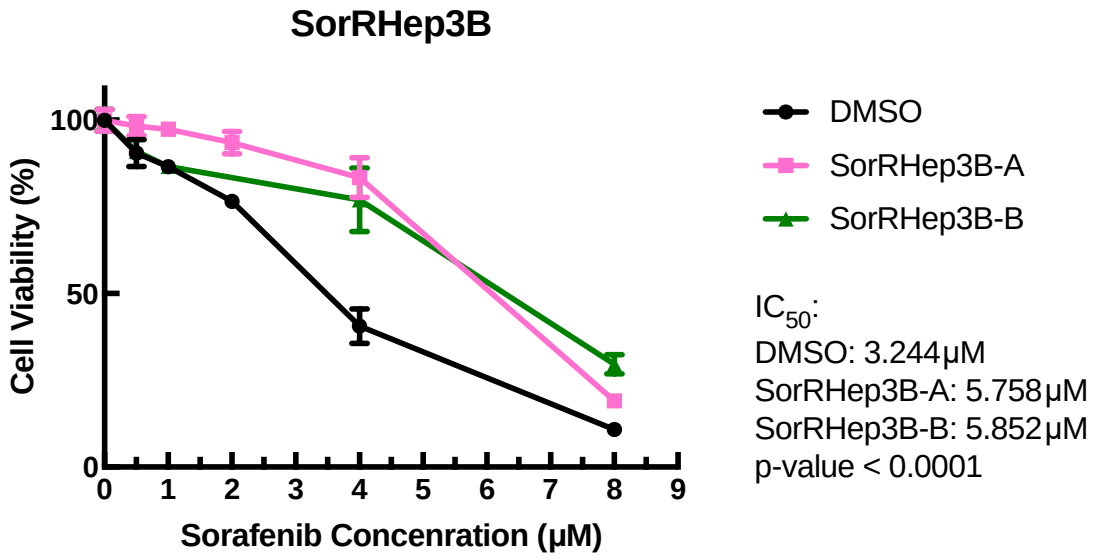


Figure 3.4: IC_{50} of SorRHep3B cells. Control cells (DMSO) and SorRHep3B cells were seeded with the same cell number and exposed to different concentrations of Sorafenib Their viability is measured with Cell Titer Glo assay after 72 hours. The experiment has been performed with triplicates.

Since SorHep3B cells have much bigger size as compared to the control ones, we wanted to determine the IC50 of 48h instead of 72h to rule out the possibility of cell death due to being over confluent. Again we obtained the IC_{50} of SorRHep3B-A cells is of 5.765 whereas that of DMSO-treated cells is 3.604 (Figure 3.5).

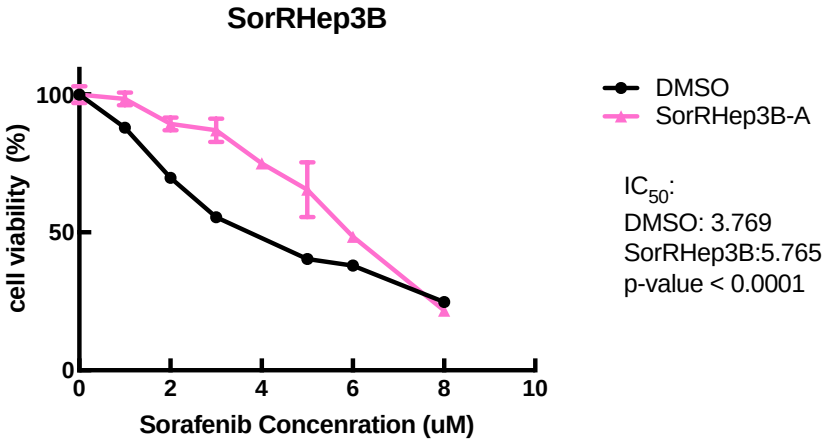


Figure 3.5: IC_{50} of SorRHep3B cells are higher than that of control cells in 48 hours. Control cells and SorRHep3B-A cells are treated with increasing Sorafenib concentrations for 48 hours and the cells viability is measured with Cell Titer Glo assay. Experiment was done with triplicate for each concentration.

3.2 Characterization of SorRHep3B cell lines:

3.2.1 Protein and mRNA expression is different between SorRHep3B and DMSO cells:

After the resistant cells were established, the differences in gene signatures were assessed with Western Blot analysis. Clear differences between the control cells and the SorRHep3B cells were observed (Figure 3.6). First of all, there was a significant downregulation of IKBKE. Correspondingly, a decrease in TBK1 was also observed at protein levels. There is an expected increase in the p-AKT levels of SorRHep3B-A as p-AKT is shown to be involved in the generation of drug resistance [18]. As mentioned above, SorRHep3B cells resembled mesenchymal cells. Hence, EMT markers were also checked. Expectedly, there was an increase in the Vimentin levels. However, E-Cadherin levels were also increased while the N-Cadherin levels were decreased in SorRHep3B cells. Since SorRHep3B cells had long branches when compared to control cells (Figure 3.3), the Integrin-α levels were also checked and found to be increased.

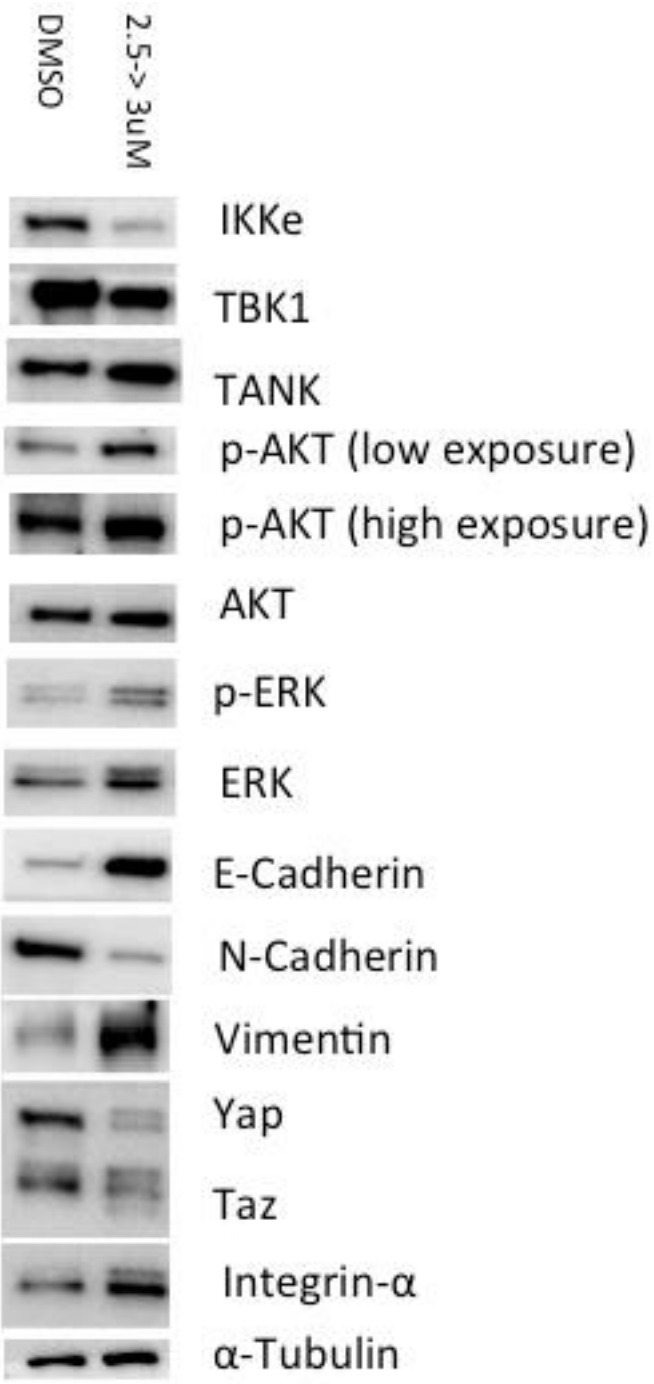


Figure 3.6: Protein expression is different DMSO-treated cells and SorRHep3B cells. Protein levels are determined by western blotting. α-tubulin is used as loading control.

In order to check the RNA levels of EMT markers, qPCR was performed. Mostly, the differences that were seen in the protein levels were mirrored also to RNA levels and similar differences were observed. IKBKE and CDH2 (N-Cadherin) transcripts were decreased by 80%. Expectedly, CDH1 (E-Cadherin) transcript was increased 2.5-fold.

However, a 65% decrease in the Vimentin RNA levels were observed. This is the exact opposite of what was seen with western blot analysis. Finally, a 200-fold increase was seen in Snai2 RNA levels in SorRHep3B cells when compared to control cells (Figure 3.7).

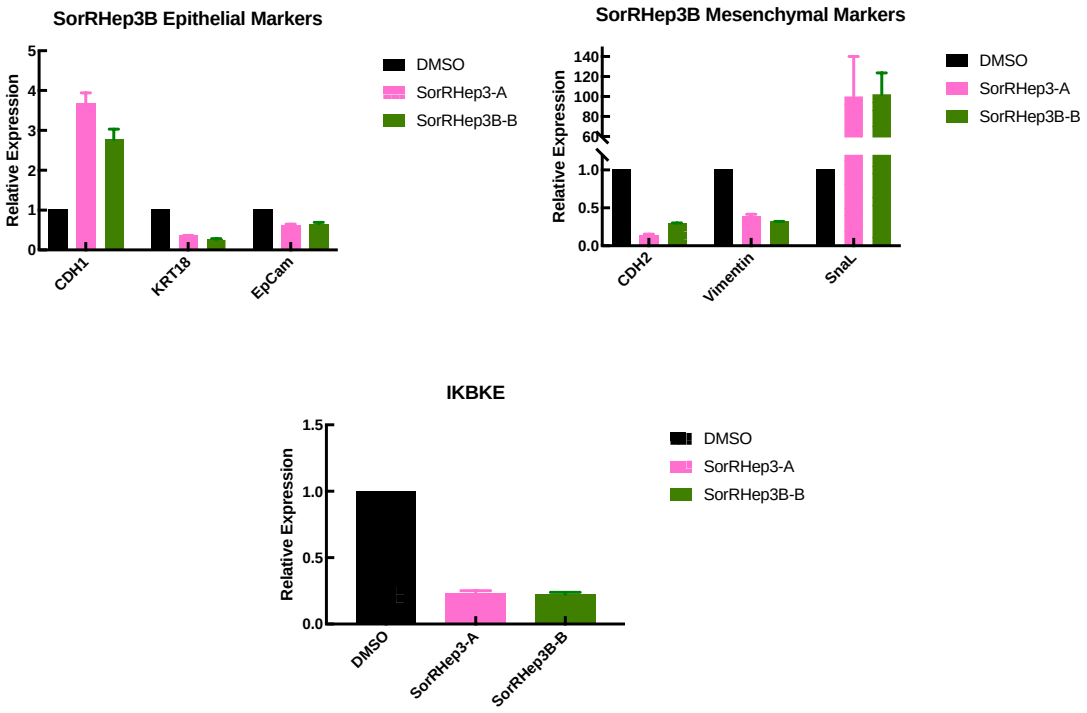


Figure 3.7: mRNA level expression is different DMSO-treated and SorRHep3B cells. A. All the epithelial markers, except CDH1 (E-Cadherin), are decreased. B. All the mesenchymal markers, expect CHD2 (N-Cadherin), are increased. C. IKBKE is decreased.

3.2.2 Assessment of proliferation of SorRHep3B cell line by Real Time Cell Analysis:

In order to assess the differences of proliferation between the control cells and SorRHep3B cells, Real Time Cell Analysis (RTCA) was used. Upon seeding 7500 cells to each well of an E-Plate, proliferation was measured. As it can be seen form the figure, SorRHep3B cells were significantly more proliferative than then control

cells. At the end of 48 hours, SorRHep3B cells proliferated almost twice as much as the control cells (Figure 3.8).

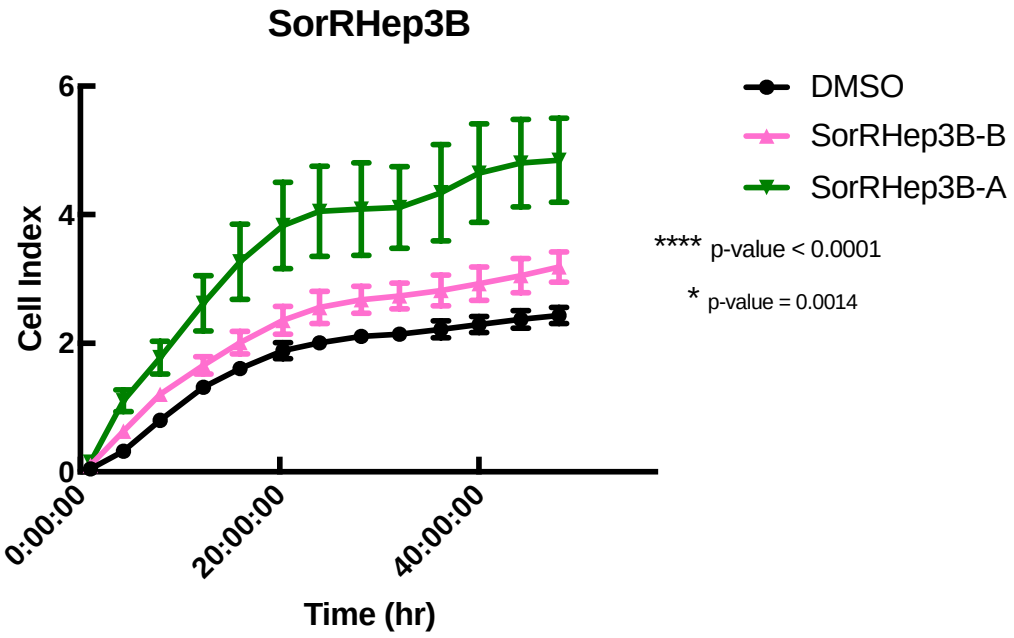


Figure 3.8: SorRHep3B cells are proliferating more than the control cells as shown by RTCA experiment. The experiment was performed as duplicates for each cell line.

3.2.3 SorRHep3B cells have survival advantage when treated with poly(I:C):

poly(I:C) is a dsRNA analog which is a ligand for TLR3. Since the IKBKE is significantly decreased in SorRHep3B, it is expected that the SorRHep3B cells to have an advantage over DMSO cells when treated with poly(I:C). As expected, the SorRHep3B cells had a significant survival advantage over DMSO-treated cells (cell viability 80% and 71% 24hr after poly(I:C) treatment, respectively. p-value = 0.0394, Figure 3.9).

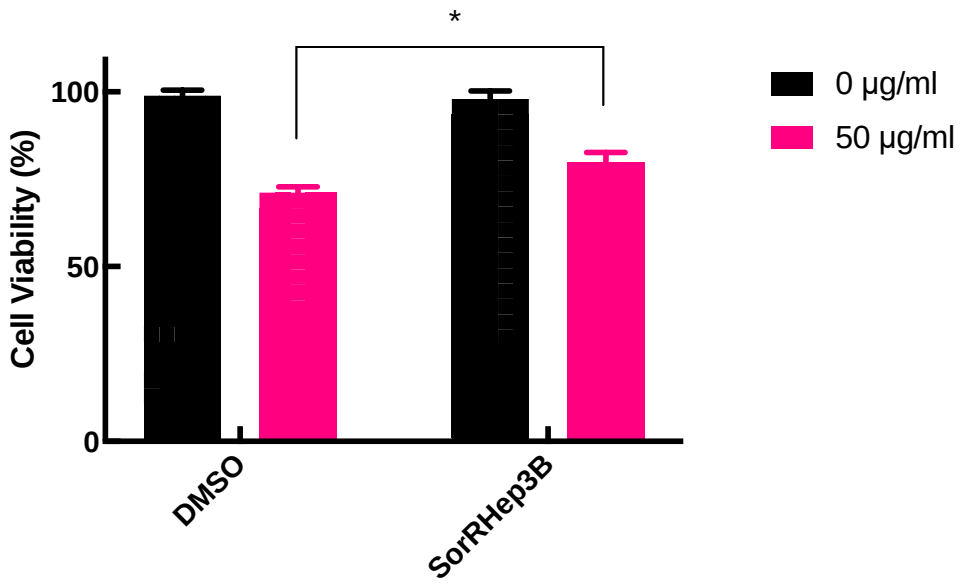


Figure 3.9: SorRHep3B cells show survival advantage upon poly(I:C) treatment. Cells are treated with 0ug/ml and 50µg/ml poly(I:C) for 24 hours and the cell viability is measured using Cell Titer Glo assay. (p-value= 0.0394). The experiment was performed twice with triplicates for each condition.

3.2.4 SorRHep3B cells show increased survival upon TGF-β treatment:

TGF-β is an inducer of EMT and since the SorRHep3B cells showed more mesenchymal properties it is expected that they would have survival advantage when treated with TGF-β. To test this, DMSO-treated control cells and SorRHep3B-A cells were treated with 5ng/ml TGF-β for 48 hours and their viability was measured with Cell Titer Glo Assay. As Figure 3.10 shows, SorRHep3B cells showed higher survival upon TGF-β treatment when compared to DMSO-treated cells. DMSO-treated cells could not increase their viability upon TGF-β treatment whereas the SorRHep3B cells had an 13% increase upon induction (p-value = 0.0184, Figure 3.10)

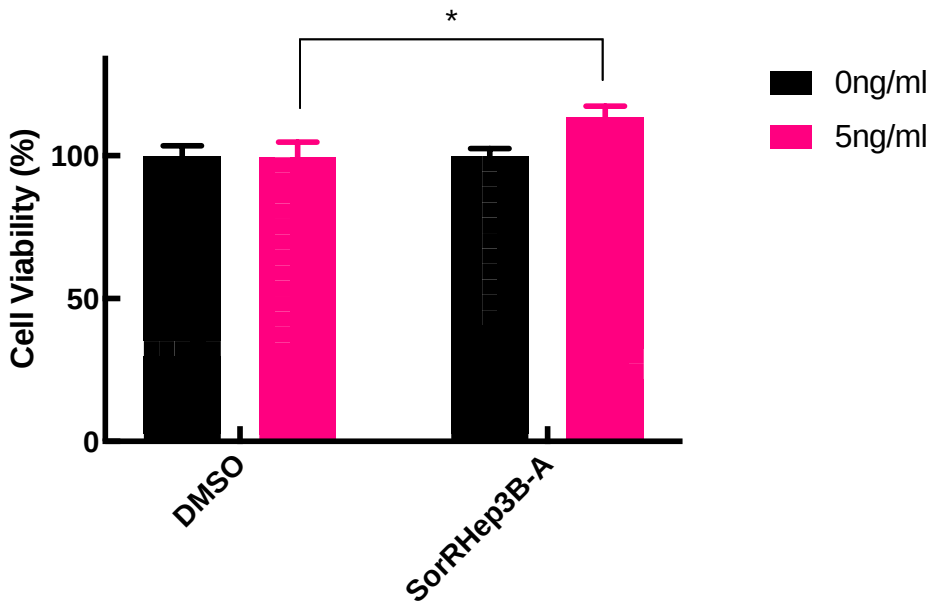


Figure 3.10: SorRHep3B cells have increased survival upon TGF-β treatment. Cells are treated with 0ng/ml and 5ng/ml TGF-b and the cell survival was assessed with Cell Titer Glo assay. The experiment was performed twice with triplicates for each condition.

3.2.5 IKBKE rescue resensitizes SorRHep3B cells to Sorafenib:

Since SorRHep3B cells have a downregulation of IKKε, we wanted to see if IKBKE rescue would re-sensitize SorRHep3B cells to Sorafenib. To this extend, IKBKE was overexpressed and treated with 4μM Sorafenib for 24 hours. Cells were seeded to 96-well plates to 70% confluent the next day. As it can be seen from Figure 3.11A that the Wild Type (WT) IKBKE rescue results in a significant decrease in cell survival. On the other hand, when kinase-dead IKBKE (KD) is exogenously expressed in the cells, it fails to induce further cell death upon Sorafenib treatment. Figure 3.11B shows the transfection of IKBKE plasmid was successful. The reason why IKBKE levels are lower in SorRHep3B cells might be because the cells are trying to degrade the protein as it is being produced.

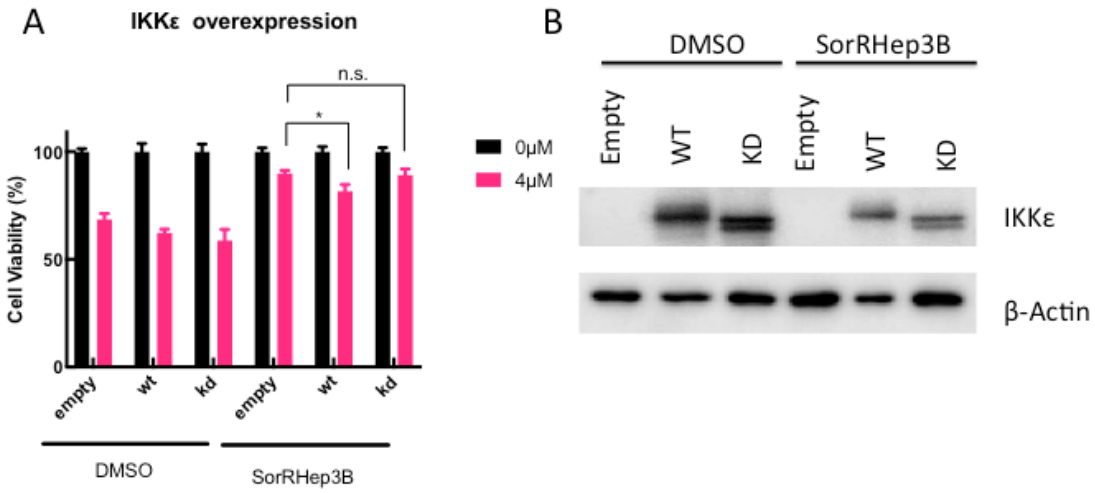


Figure 3.11: IKBKE rescue re-sensitizes SorRHep3B cells to Sorafenib. A. DMSO or SorRHep3B cells are transfected with empty pcDNA vector (Empty), IKBKE wild Type (WT) plasmid or Kinase Dead IKBKE (KD) plasmid and treated with 4uM Sorafenib for 24 hours. Cell viability is assessed with Cell Titer Glo assay. B. IKBKE levels are checked with western blotting to confirm that the transfection is successful. β-Actin is used as loading control. The experiment was performed twice, with either triplicates or 5-replicates for each condition. The image is representative of both experiments. (p-value: 0.0159).

3.3 Hep3B cells with IKKε loss of function show some similar phenotypes to SorHep3B cells

3.3.1 IKBKE depletion confers Sorafenib resistance to naïveHep3B

Since the level IKBKE expression is dramatically decreased in SorRHep3B cells, we would like to test the Sorafenib resistance ability of naïve Hep3B cells upon IKBKE depletion by shRNA. As expected, IKBKE depleted Hep3B cells, appeared to be

more resistant to Sorafenib. First of all, the Sorafenib IC_{50} of the depleted cells were significantly higher than the control cells (Figure 3.12).

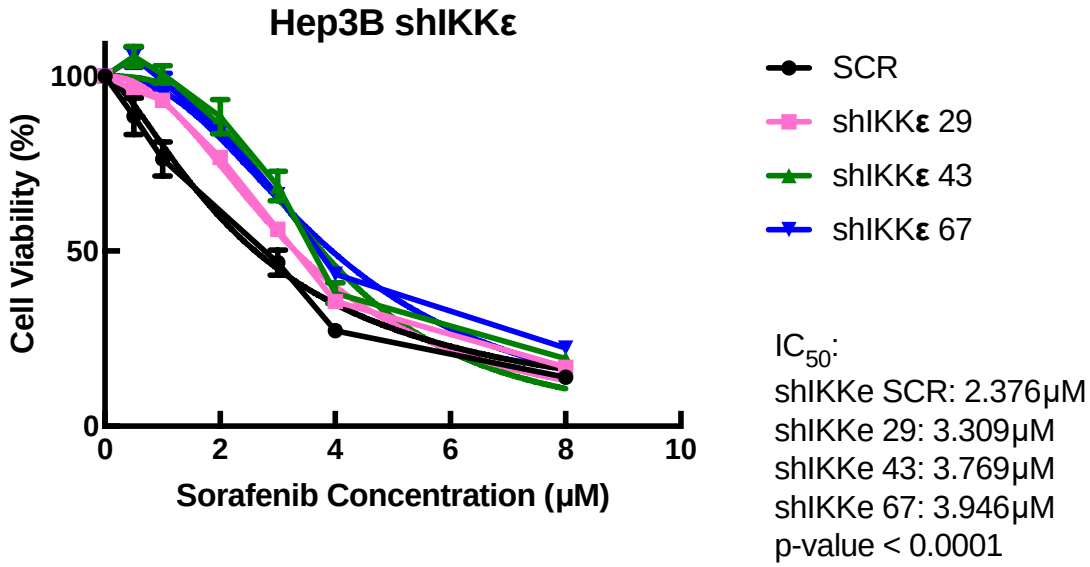


Figure 3.12: Depletion of IKBKE with shRNA increases resistance of Hep3B cells to Sorafenib. Cells are treated with different concentrations of Sorafenib for 72 hours and their viability is measured with Cell Titer Glo assay. The experiment was performed twice with triplicates for each condition.

In addition, IKBKE depleted cells were treated with 7μM Sorafenib and their apoptosis was quantified by measuring the Caspase 3/7 activation of the cells using Caspase3/7 Glo assay. Figure 3.13 shows that the shSCR control cells went through apoptosis almost twice as much as the shIKKε cells upon Sorafenib.

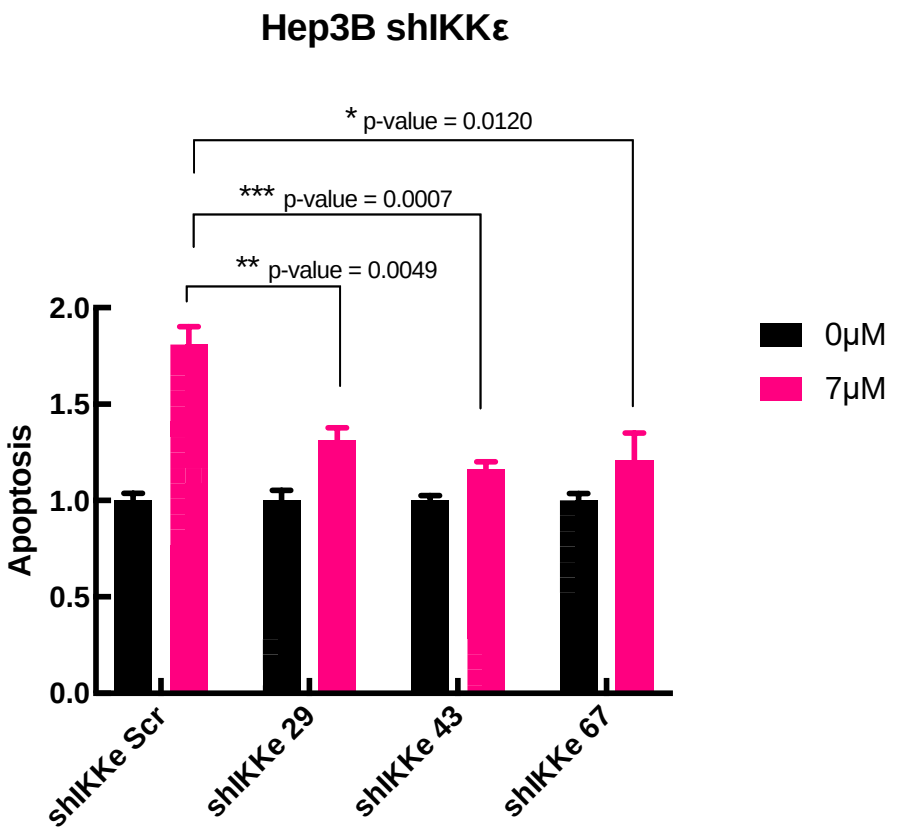


Figure 3.13: IKBKE depleted cells do not go through apoptosis upon Sorafenib treatment. shSCR and shIKKε cells are treated with 0μM and 7μM Sorafenib for 48 hours and their apoptosis was measured using Caspase 3/7 Glo assay. The experiment was performed with triplicates for each condition.

3.4 IKBKE inhibition on naïve Hep3B cells also render them more resistant to Sorafenib:

3.4.1 Amlexanox:

Amlexanox is a kinase inhibitor of IKBKE. We would like to test if kinase activity of IKKε is involved in Sorafenib resistance of naïveHep3B. To do this, naïve Hep3B cells were treated with only Sorafenib or Sorafenib/Amlexanox combination and their viability was measured after 72 hours. As it can be seen from the Figure 3.14, the IC_{50} value of naïve cells were increased significantly upon 50μM Amlexanox treatment: 2.5 for 0μM Amlexanox and 3.1 for 50μM Amlexanox (p-value < 0.0001).

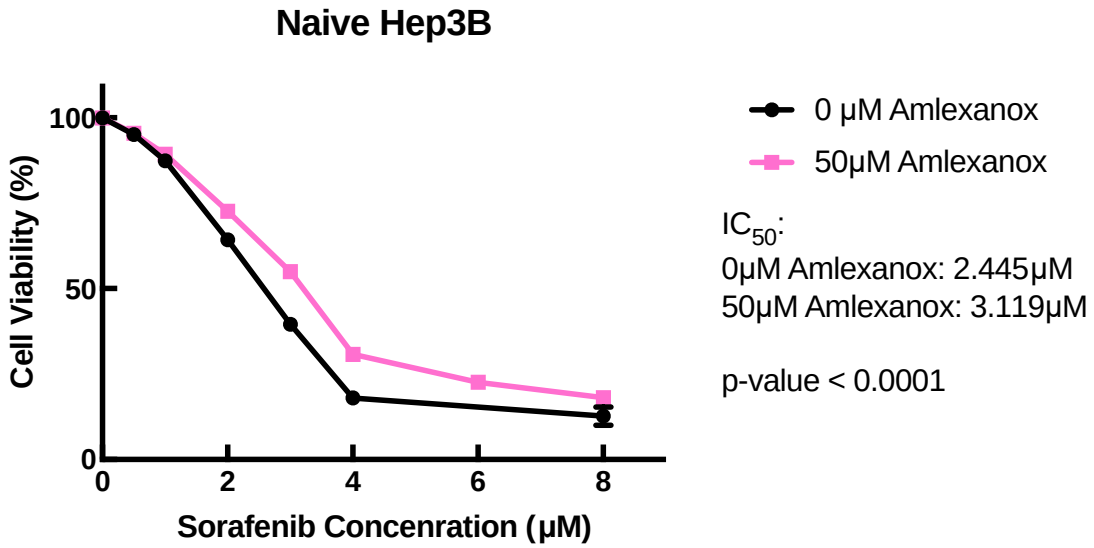


Figure 3.14: Amlexanox decreases naïve Hep3B sensitivity to Sorafenib. Naïve Hep3B cells were treated with different concentrations of Sorafenib for 72 hours and the cell viability is measured using Cell Titer Glo Assay. Experiment was performed twice with triplicate for each condition.

3.4.2 IKBKE-depleted cells are proliferating more than the control cells:

Proliferative abilities of the IKBKE-depleted cells were assessed using RTCA. Upon seeding the cells, the cell index was measured every 2 hours for 36 hours. Figure 3.15 shows that the depleted cells were proliferating significantly more than the control cells.

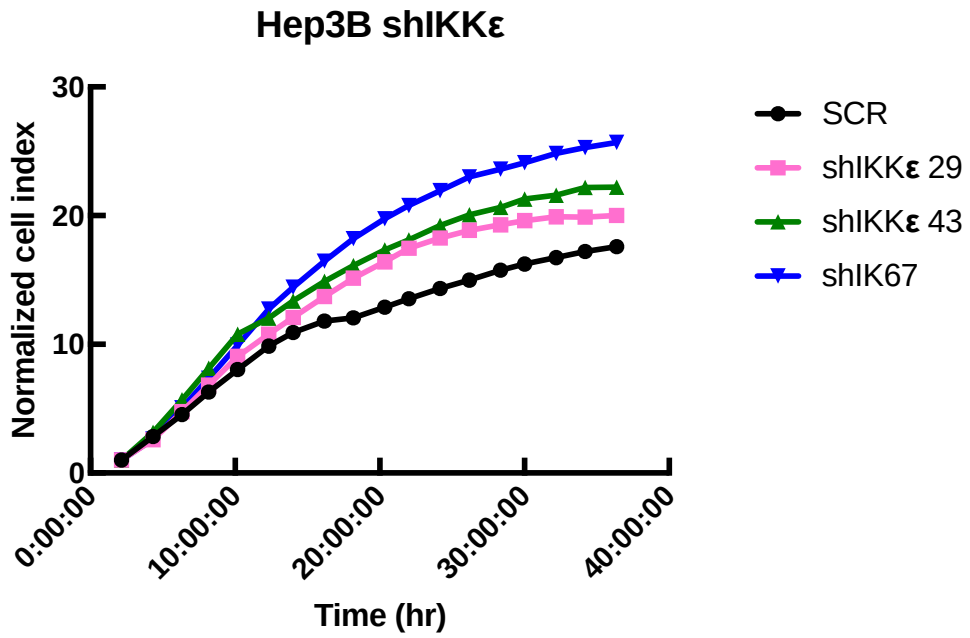


Figure 3.15 IKBKE-depleted cells are proliferating more than the control cells. Cells are seeded to e-plate and the cell proliferation is measured with RTCA. The experiment was performed as duplicates for each cell line.

3.4.3 IKBKE rescue in depleted Hep3B re-sensitizes them to Sorafenib again:

As the IKBKE-depleted cells appear to be resistant to Sorafenib, it is expected that IKBKE rescue might re-sensitize the cells to Sorafenib. To test this, naïveHep3B cells with IKBKE depletion were seeded to 96 well plate transfected with IKKε plasmid and treated with 4μM Sorafenib. On the contrary to expectation, a significant difference was not seen between IKBKE rescue and the empty vector control, however, a trend can be seen (Figure 3.16).

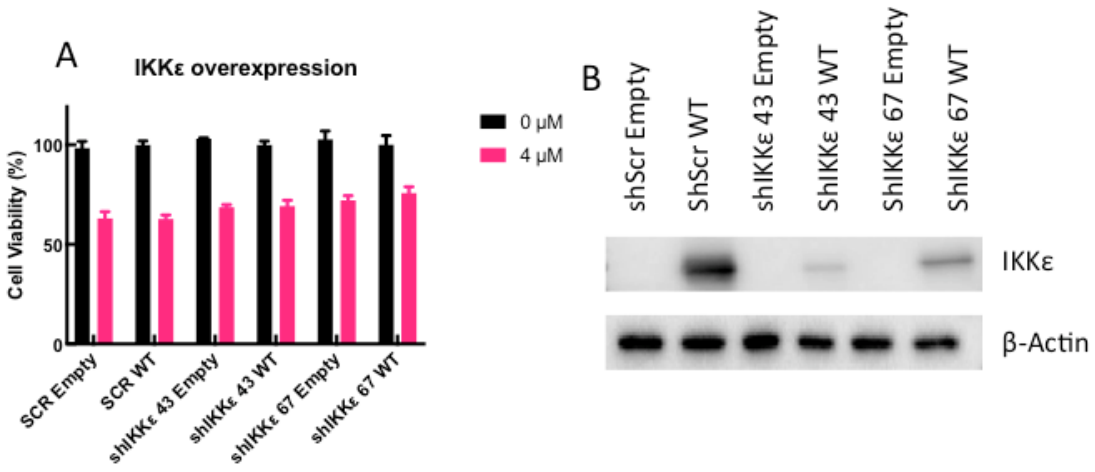


Figure 3.16: IKBKE rescue on shRNA-IKBKE depleted naïve cells. A. Cells were transfected with either empty pcDNA vector (Empty) or wild type IKBKE plasmid (WT) and treated with 4μM Sorafenib for 24 hours. Cell viability was measured by Cell Titer Glo Assay. B. IKBKE levels are tested with western blotting to check transfection. β-Actin is used as loading control. The experiment was performed for 5 replicates for each condition.

4 Discussion:

Currently, Sorafenib is the only drug approved by FDA as a first-line treatment option for patients with advanced Hepatocellular Carcinoma (HCC). However, Sorafenib can only increase the overall survival of the patients by approximately 4 months before resistance occurs. Many studies have been conducted to elucidate the resistance mechanism and to find a way to overcome resistance, yet the complete picture is still missing. In this study, a novel resistance mechanism which involves IKBKE is hypothesized. To this extend, Sorafenib resistant Hep3B cell line (SorRHep3B,) IKKε depleted Hep3B cell line and Amlexanox, a small molecule inhibitor of IKBKE, on naïve Hep3B cell line were utilized. This study suggests a novel role of IKKε, a protein that was previously thought to have oncogenic functions, in Sorafenib resistance mechanism in HCC

4.1 IKKε involvement in Sorafenib resistance of Hep3B cell line:

IKBKE has been shown to have oncogenic functions and is overexpressed in many cancer types including breast cancer [35], esophageal squamous cell carcinomas [36], glioma [37] and ovarian cancer [38], colon cancer [43], gastric cancer [44], melanoma [45], pancreatic cancer [46], prostate cancer [47], renal cancer [48] and lung cancer [49]. In addition, IKBKE has been implicated to take part in drug resistance, such as cisplatin-resistance in ovarian cancer [38]. However, there is no documented correlation between IKBKE expression level and Sorafenib resistance. This study showed a novel, unexpected relationship between IKBKE expression and Sorafenib resistance. On contrary to the

proposed oncogenic properties of IKKε, here we have strong evidence showing that the decreased expression of IKBKE is associated with resistance against Sorafenib in one of the HCC cell lines.

4.1.1 Loss of IKKε is associated with increase resistance of Hep3B to Sorafenib as reflected by IC_{50} value and Caspase 3/7 activity:

This study focused on Sorafenib resistance in Hep3B where IKKε expression level was the highest among all HCC cell lines that we tried (Appendix). Sorafenib-resistant Hep3B cell lines (SorRHep3B) were created through a continuous exposure of naïve Hep3B cells to drug over time. Upon creation of SorRHep3B cell line, it was observed that IKBKE expression, at both protein level and mRNA level, is nearly ablated in Sorafenib resistant cells when compared to their naïve counterpart (Figure 3.6 and Figure 3.7). This phenomenon is further supported by the loss of function of IKKε by shRNA and the inhibition of IKBKE using a small molecule inhibitor, Amlexanox on naïve Hep3B cells. When Hep3B cells devoid of IKKε or having IKKε inhibited by a specific inhibitor, this HCC cell line exhibits a significant resistance against Sorafenib, reflected by the IC_{50} values (Figure 3.12 and Figure 3.14). All three approaches suggested that IKKε is definitely involved in Sorafenib resistance in Hep3B

Sorafenib is an anti-proliferative agent that induces apoptosis [13]. This is observed in Hep3B-shIKBKE cells that are treated with Sorafenib. Control cells showed significantly higher Caspase 3/7 activity when compared to depleted

cells (Figure 3.13). The fact that IKBKE-depletion itself could inhibit Sorafenib-induced apoptosis in HCC cells shows dependence of these cells to the loss of IKBKE for the maintenance of resistance. In addition, in Hep3B-shIKBKE-67 cells, no significant increase in Caspase 3/7 activity upon Sorafenib treatment is observed. Concordantly, this cell line is the one with highest Sorafenib IC_{50} with a very good depletion. This suggests that IKBKE-loss plays a crucial role in inhibiting the pro-apoptotic role of Sorafenib.

4.1.2 IKKε rescue resensitizes IKKε deficient Hep3B cells to Sorafenib:

To further explore the role of IKBKE in the acquisition and maintenance of Sorafenib resistance in Hep3B cells, IKBKE was recued in SorRHep3B cells. The exogenous IKBKE expression re-sensitized the SorRHep3B cells to Sorafenib and significantly decreased cell survival upon Sorafenib treatment. Expectedly, when SorRHep3B cells were transfected with a kinase-dead version of IKBKE, the resensitization is lost (Figure 3.11). This emphasizes the importance of functional IKBKE protein for Sorafenib response. A similar decrease in cell viability was observed upon Sorafenib treatment of Hep3B-shIKBKE cells following IKBKE rescue. Although this decrease was not significant, a trend could be observed (Figure 3.16). The fact that both of these cell lines show a similar response to exogenous IKBKE expression elucidates the involvement of IKBKE in Sorafenib resistance. Moreover, the fact that rescuing IKBKE in Hep3B-shIKBKE cells confers them to become more responsive to Sorafenib suggests that there might be a direct relationship between the loss of IKBKE and the acquisition and maintenance of Sorafenib resistance in Hep3B cells. Last but not least, the

SorRHep3B cells and Hep3B-shIKBKE showed less IKBKE expression even after the transfection with the IKBKE plasmid. This might be due to the fact that the cells were still trying to downregulate the expression of IKBKE. This is another factor that suggests the importance of IKBKE involvement in Sorafenib resistance. However, to definitely conclude that the re-sensitization of SorRHep3B cells and Hep3B-shIKBKE cells against Sorafenib is caused by IKBKE expression, the expression should be stable enough. In other words, if the cells are depleting the protein as the exogenous protein is expressed, the effect of this rescue might be impaired. Thus, the actual effect of IKBKE rescue on Sorafenib response might be underestimated.

4.1.3 Loss of IKKε is associated with increased cell growth as seen both in SorHep3B and IKKε depleted Hep3B.

The reduced sensitivity of IKKε depleted cells against Sorafenib was accompanied by increased proliferation, as seen in SorRHep3B cells. (Figure 3.8 and Figure 3.15). These results provide additional proof that IKBKE loss confers a more aggressive Sorafenib resistance of this HCC cell line. In a separate study ongoing in our lab, we observed that mice injected with Hep3B-shIKBKE cells show bigger tumor volume than control cells. This is in parallel with the findings of this study, showing that the Hep3B-shIKBKE cells that are more resistant to Sorafenib are also more proliferative than and more aggressive than the control counterparts.

4.1.4 Loss of IKKε protects Hep3B against apoptosis by TGF-β or cell insult such as poly(I:C) as seen in SorHep3B cells.

When SorRHep3B cells and Hep3B-shIKBKE cells are treated with poly(I:C), a significant increase in the cell survival is seen (Figure 3.9). This suggests a protective role of IKKε against insults in SorRHep3B cells. This protective role is also seen when the SorRHep3B cells are treated with TGF-β. Upon 48-hour treatment, a significant increase in the cell proliferation is observed in SorRHep3B cells when compared to DMSO-treated cells (Figure 3.10). Taken together, this further exemplifies the protective role of IKKε loss on Sorafenib resistance.

4.2 SorHep3B exhibits partial EMT features.

In addition to unresponsiveness against Sorafenib, SorRHep3B cells showed distinct morphological changes from the control (DMSO-treated) cells. First of all, the SorRHep3B cells resemble mesenchymal cells (even though naïve Hep3B cells originates from epithelial cells). SorRHep3B cells were markedly bigger than the controls. Moreover, resistant cells had long branches, which are generally associated with mesenchymal cells (Figure 3.3). This is in accordance with the fact that Sorafenib resistant cells show higher metastatic abilities than their sensitive counterparts [40]. Surprisingly, despite a pronounced level of Vimentin, α-integrin (prominent mesenchymal markers), our SorHep3B has increased E-cadherin, (a classical epithelial marker), and decreased N- Cadherin, (an classical mesenchymal marker) (Figure 3.6). However, literature has

documented that complete Epithelial-to-Mesenchymal Transition (EMT) is not always seen in cancer samples and this so-called “partial EMT phenotype” is involved in more aggressive tumors [50]. In a recent study by Yamashita et al show that the primary tumor samples of breast cancer tumors and lymph metastasis show co-expression of both E-Cadherin and Vimentin. They argue that the partial EMT phenotype seen is associated with more aggressive tumors [41]. Moreover, cancer cells that show both epithelial and mesenchymal features can leave the bloodstream more efficiently and show higher metastatic ability than completely mesenchymal cells [42]. This unique expression pattern might explain the fast rate of resistance development. To our knowledge, there are no studies showing this unusual trait of Sorafenib resistant HCC cells. This particular expression pattern of SorRHep3B cell line together with a marked decrease of IKKε expression level may shed light on the Sorafenib resistance mechanisms in HCC. To verify if this phenomenon would be also common in other HCC cell lines, Sorafenib resistant HepG2 cell lines are being created in our lab.

5 Conclusion and Future Perspectives:

Sorafenib is the only drug used as a first line therapeutic agent in advanced HCC and unfortunately, resistance develops very quickly, hampering the benefit patients can get from the drug. This study aimed to find a possible mechanism to overcome Sorafenib resistance. To this extend, Sorafenib resistant SorRHep3B cell lines were established and the mechanisms for their resistance were investigated. At the end of the study, IKBKE has been found to be involved in the acquisition of Sorafenib resistance in HCC cell line Hep3B. Our studies concluded that the loss of IKBKE in the cells confers them to a more resistant phenotype and helped to maintain the resistance of the cells.

As a part of the study, IKBKE was rescued in SorRHep3B cells and Hep3B-shIKBKE cells. Although the transfection was successful, the expression levels of the exogenous IKBKE levels were much lower in SorRHep3B cells and Hep3B-shIKBKE cells (when compared to DMSO-treated and Hep3B-shScr cells, respectively). We concluded that the cells tried to deplete the IKBKE protein even after the forced expression. As mentioned above, this might result in the underestimation of the real role IKBKE plays in Sorafenib resistance since the actual effect of the protein cannot be observed. In other words, IKBKE is being produced and degraded simultaneously. To bypass this effect, the experiments should be performed in the presence of protein degradation inhibitors. This would result in IKBKE showing complete effect in the cell.

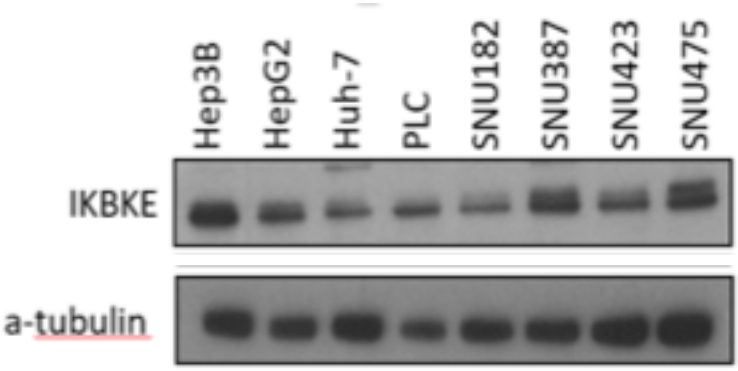
During the scope of the study, SorRHep3B cells and Hep3B-shIKBKE cells were only transiently transfected with IKBKE to rescue its expression; stable transfection and stable expression of IKBKE in SorRHep3B cells might be utilized to see the long-term effects of IKBKE expression on Sorafenib resistance. This might be beneficial especially in clinical settings to see if the induced IKBKE expression can have an effect for patients who are not responsive to Sorafenib.

IKBKE is a protein that plays important oncogenic roles in other types of cancer but this is the first study that suggests it can also have a tumor suppressor role. This study might be a basis for studying the roles of IKBKE in other types of cancer. In addition, investigating the role IKBKE at different might worthwhile since it may play dual roles in different stages of cancer or in different cancer types.

Equally importantly, the mechanisms by which IKBKE contributes to Sorafenib resistance should be investigated in depth. Clarifying the mechanism of resistance would be of tremendous benefit in generating therapeutic options for patients who have progressed on Sorafenib. In order to do so, new Sorafenib resistant cell lines are being created by our lab members. First of all, a new Sorafenib-resistant Hep3B cells is underway, this time increasing the Sorafenib concentration softer. In addition, generation of Sorafenib-resistant HepG2 cells is ongoing. Showing that the effects that we get is not cell-type dependent and dose-dependent will we very beneficial for further studies of trying to overcome Sorafenib resistance in HCC.

6 APPENDIX:

Hep3B cells are chosen to generate Sorafenib resistant cells since these cells had the most IKBKE expression among all the other cell lines tried. Figure 6.1 shows the IKBKE levels of difference cell lines.



Supplemented Figure 1: Different cell lines have different basal IKBKE expression.

7 REFERENCES:

1 Who.int. (2018). WHO | Key statistics. [online] Available at: http://www.who.int/cancer/resources/keyfacts/en/ [Accessed 17 Jun. 2018].

2 M. Ervik, F. Lam, J. Ferlay, L. Mery, I. Soerjomataram, F. Bray (2016). Cancer Today. Lyon, France: International Agency for Research on Cancer. Cancer Today. Available from: http://gco.iarc.fr/today, [accessed 17/06/2018].

3 Llovet, J., Zucman-Rossi, J., Pikarsky, E., Sangro, B., Schwartz, M., Sherman, M. and Gores, G. (2016). Hepatocellular carcinoma. Nature Reviews Disease Primers, 2, p.16018.

4 Forner, A., Reig, M. and Bruix, J. (2018). Hepatocellular carcinoma. The Lancet, 391(10127), pp.1301-1314.

5 Brito, A., Abrantes, A., Tralhão, J. and Botelho, M. (2016). Targeting hepatocellular carcinoma: what did we discover so far?. Oncology Reviews, 10(2).

6 Liver transplantation for hepatocellular carcinoma: Comparison of the proposed UCSF criteria with the Milan criteria and the Pittsburgh modified TNM criteria. Liver Transplantation, 8(9), pp.765-774.

7 van Zijl, F., Zulehner, G., Petz, M., Schneller, D., Kornauth, C., Hau, M., Machat, G., Grubinger, M., Huber, H. and Mikulits, W. (2009). Epithelial–mesenchymal transition in hepatocellular carcinoma. Future Oncology, 5(8), pp.1169-1179.

8 Bai, W., Wang, Y., Zhao, Y., Qi, X., Yin, Z., He, C., Li, R., Wu, K., Xia, J., Fan, D. and Han, G. (2013). Sorafenib in combination with transarterial

chemoembolization improves the survival of patients with unresectable hepatocellular carcinoma: A propensity score matching study. Journal of Digestive Diseases, 14(4), pp.181-190.

9 Zhao, Y., Wang, W., Guan, S., Li, H., Xu, R., Wu, J., Liu, J., Li, H., Bai, W., Yin, Z., Fan, D., Zhang, Z. and Han, G. (2013). Sorafenib combined with transarterial chemoembolization for the treatment of advanced hepatocellular carcinoma: a large-scale multicenter study of 222 patients. Annals of Oncology, 24(7), pp.1786-1792.

10 Daher, S., Massarwa, M., Benson, A. and Khoury, T. (2017). Current and Future Treatment of Hepatocellular Carcinoma: An Updated Comprehensive Review. Journal of Clinical and Translational Hepatology, 6(1), pp.1-10.

11 El-Khoueiry, A., Sangro, B., Yau, T., Crocenzi, T., Kudo, M., Hsu, C., Kim, T., Choo, S., Trojan, J., Welling, T., Meyer, T., Kang, Y., Yeo, W., Chopra, A., Anderson, J., dela Cruz, C., Lang, L., Neely, J., Tang, H., Dastani, H. and Melero, I. (2017). Nivolumab in patients with advanced hepatocellular carcinoma (CheckMate 040): an open-label, non-comparative, phase 1/2 dose escalation and expansion trial. The Lancet, 389(10088), pp.2492-2502.

12 Chen, J., Jin, R., Zhao, J., Liu, J., Ying, H., Yan, H., Zhou, S., Liang, Y., Huang, D., Liang, X., Yu, H., Lin, H. and Cai, X. (2015). Potential molecular, cellular and microenvironmental mechanism of sorafenib resistance in hepatocellular carcinoma. Cancer Letters, 367(1), pp.1-11.

13 Zhu, Y., Zheng, B., Wang, H. and Chen, L. (2017). New knowledge of the mechanisms of sorafenib resistance in liver cancer. Acta Pharmacologica Sinica, 38(5), pp.614-622.

14 Llovet, J., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J., de Oliveira, A., Santoro, A., Raoul, J., Forner, A., Schwartz, M., Porta, C., Zeuzem, S., Bolondi, L., Greten, T., Galle, P., Seitz, J., Borbath, I., Häussinger, D., Giannaris, T., Shan, M., Moscovici, M., Voliotis, D. and Bruix, J. (2008). Sorafenib in Advanced Hepatocellular Carcinoma. New England Journal of Medicine, 359(4), pp.378-390.

15 Cheng, A., Kang, Y., Chen, Z., Tsao, C., Qin, S., Kim, J., Luo, R., Feng, J., Ye, S., Yang, T., Xu, J., Sun, Y., Liang, H., Liu, J., Wang, J., Tak, W., Pan, H., Burock, K., Zou, J., Voliotis, D. and Guan, Z. (2009). Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. The Lancet Oncology, 10(1), pp.25-34.

16 Cantarini, M., Trevisani, F., Morselli-Labate, A., Rapaccini, G., Farinati, F., Poggio, P., Nolfo, M., Benvegnu, L., Zoli, M., Borzio, F. and Bernardi, M. (2006). Effect of the Etiology of Viral Cirrhosis on the Survival of Patients with Hepatocellular Carcinoma. The American Journal of Gastroenterology, 101(1), pp.91-98.

17 Sun, W. and Cabrera, R. (2018). Systemic Treatment of Patients with Advanced, Unresectable Hepatocellular Carcinoma: Emergence of Therapies. Journal of Gastrointestinal Cancer, 49(2), pp.107-115.

18 Zhai, B. (2013). Mechanisms of resistance to sorafenib and the corresponding strategies in hepatocellular carcinoma. World Journal of Hepatology, 5(7), p.345.

19 Zhai, B., Hu, F., Jiang, X., Xu, J., Zhao, D., Liu, B., Pan, S., Dong, X., Tan, G., Wei, Z., Qiao, H., Jiang, H. and Sun, X. (2014). Inhibition of Akt Reverses

the Acquired Resistance to Sorafenib by Switching Protective Autophagy to Autophagic Cell Death in Hepatocellular Carcinoma. Molecular Cancer Therapeutics, 13(6), pp.1589-1598.

20 Fischer, K., Durrans, A., Lee, S., Sheng, J., Li, F., Wong, S., Choi, H., El Rayes, T., Ryu, S., Troeger, J., Schwabe, R., Vahdat, L., Altorki, N., Mittal, V. and Gao, D. (2015). Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance. Nature, 527(7579), pp.472-476.

21 Zheng, X., Carstens, J., Kim, J., Scheible, M., Kaye, J., Sugimoto, H., Wu, C., LeBleu, V. and Kalluri, R. (2015). Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer. Nature, 527(7579), pp.525-530.

22 Jing Wang, Qiang Wei, Xin Wang, Shengli Tang, Hao Liu, Fugui Zhang, Maryam K. Mohammed, Jiayi Huang, Dan Guo, Minpeng Lu, Feng Liu, Jianxiang Liu, Chao Ma, Xue Hu, Rex C. Haydon, Tong-Chuan He, Hue H. Luu.Transition to resistance: An unexpected role of the EMT in cancer chemoresistance,Genes & Diseases,Volume 3, Issue 1,2016,Pages 3-6,ISSN 2352-3042,https://doi.org/10.1016/j.gendis.2016.01.002

23 Raoul, J., Kudo, M., Finn, R., Edeline, J., Reig, M. and Galle, P. (2018). Systemic therapy for intermediate and advanced hepatocellular carcinoma: Sorafenib and beyond. Cancer Treatment Reviews, 68, pp.16-24.

24 Grigore, A., Jolly, M., Jia, D., Farach-Carson, M. and Levine, H. (2016). Tumor Budding: The Name is EMT. Partial EMT. Journal of Clinical Medicine, 5(5), p.51.

25 Giannelli, G., Koudelkova, P., Dituri, F. and Mikulits, W. (2016). Role of epithelial to mesenchymal transition in hepatocellular carcinoma. Journal of Hepatology, 65(4), pp.798-808.

26 Liu, X., Yun, F., Shi, L., Li, Z., Luo, N. and Jia, Y. (2015). Roles of Signaling Pathways in the Epithelial-Mesenchymal Transition in Cancer. Asian Pacific Journal of Cancer Prevention, 16(15), pp.6201-6206.

27 Malfettone, A., Soukupova, J., Bertran, E., Crosas-Molist, E., Lastra, R., Fernando, J., Koudelkova, P., Rani, B., Fabra, Á., Serrano, T., Ramos, E., Mikulits, W., Giannelli, G. and Fabregat, I. (2017). Transforming growth factor-β-induced plasticity causes a migratory stemness phenotype in hepatocellular carcinoma. Cancer Letters, 392, pp.39-50.

28 Wu, Y. and Zhou, B. (2010). TNF-α/NF-κB/Snail pathway in cancer cell migration and invasion. British Journal of Cancer, 102(4), pp.639-644.

29 Zhang, P., Li, K., Shen, Y., Gao, P., Dong, Z., Cai, J., Zhang, C., Huang, X., Tian, M., Hu, Z., Gao, D., Fan, J., Ke, A. and Shi, G. (2016). Galectin-1 induces hepatocellular carcinoma EMT and sorafenib resistance by activating FAK/PI3K/AKT signaling. Cell Death & Disease, 7(4), pp.e2201-e2201.

30 Ncbi.nlm.nih.gov. (2018). IKBKE inhibitor of nuclear factor kappa B kinase subunit epsilon [Homo sapiens (human)] - Gene - NCBI. [online] Available at: https://www.ncbi.nlm.nih.gov/gene/9641 [Accessed 21 Jun. 2018].

31 Chau, T., Gioia, R., Gatot, J., Patrascu, F., Carpentier, I., Chapelle, J., O'Neill, L., Beyaert, R., Piette, J. and Chariot, A. (2008). Are the IKKs and IKK-related kinases TBK1 and IKK-ε similarly activated?. Trends in Biochemical Sciences, 33(4), pp.171-180.

32 Guo, J., Tian, W., Shu, S., Xin, Y., Shou, C. and Cheng, J. (2013). IKBKE Phosphorylation and Inhibition of FOXO3a: A Mechanism of IKBKE Oncogenic Function. PLoS ONE, 8(5), p.e63636.

33 Panday, A., Inda, M., Bagam, P., Sahoo, M., Osorio, D. and Batra, S. (2016). Transcription Factor NF-κB: An Update on Intervention Strategies. Archivum Immunologiae et Therapiae Experimentalis, 64(6), pp.463-483.

34 Luedde, T. and Schwabe, R. (2011). NF-κB in the liver—linking injury, fibrosis and hepatocellular carcinoma. Nature Reviews Gastroenterology & Hepatology, 8(2), pp.108-118.

35 Shen, R., Zhou, A., Kim, E., Lim, E., Habelhah, H. and Hahn, W. (2012). I B Kinase Phosphorylates TRAF2 To Promote Mammary Epithelial Cell Transformation. Molecular and Cellular Biology, 32(23), pp.4756-4768.

36 Kang, M., Kim, M., Kim, S., Ahn, C., Yoo, N. and Lee, S. (2009). NF-κB signalling proteins p50/p105, p52/p100, RelA, and IKKε are over-expressed in oesophageal squamous cell carcinomas. Pathology, 41(7), pp.622-625.

37 Guan, H., Zhang, H., Cai, J., Wu, J., Yuan, J., Li, J., Huang, Z. and Li, M. (2010). IKBKE is over-expressed in glioma and contributes to resistance of glioma cells to apoptosis via activating NF-κB. The Journal of Pathology, 223(3), pp.436-445.

38 Guo, J., Shu, S., He, L., Lee, Y., Kruk, P., Grenman, S., Nicosia, S., Mor, G., Schell, M., Coppola, D. and Cheng, J. (2009). Deregulation of IKBKE Is Associated with Tumor Progression, Poor Prognosis, and Cisplatin Resistance in Ovarian Cancer. The American Journal of Pathology, 175(1), pp.324-333.

39 Chow, A., Ng, L., Lam, C., Wong, S., Wan, T., Cheng, N., Yau, T., Poon, R. and Pang, R. (2013). The Enhanced Metastatic Potential of Hepatocellular Carcinoma (HCC) Cells with Sorafenib Resistance. PLoS ONE, 8(11), p.e78675.

40 Yamashita, N., Tokunaga, E., Iimori, M., Inoue, Y., Tanaka, K., Kitao, H., Saeki, H., Oki, E. and Maehara, Y. (2018). Epithelial Paradox: Clinical Significance of Coexpression of E-cadherin and Vimentin With Regard to Invasion and Metastasis of Breast Cancer. Clinical Breast Cancer.

41 Saitoh, M. (2018). Involvement of partial EMT in cancer progression. The Journal of Biochemistry.

42 Takeda, K. and Akira, S. (2004). TLR signaling pathways. Seminars in Immunology, 16(1), pp.3-9.

43 Goktuna, S., Shostak, K., Chau, T., Heukamp, L., Hennuy, B., Duong, H., Ladang, A., Close, P., Klevernic, I., Olivier, F., Florin, A., Ehx, G., Baron, F., Vandereyken, M., Rahmouni, S., Vereecke, L., van Loo, G., Bu ttner, R., Greten, F. and Chariot, A. (2016). The Prosurvival IKK-Related Kinase IKK Integrates LPS and IL17A Signaling Cascades to Promote Wnt-Dependent Tumor Development in the Intestine. Cancer Research, 76(9), pp.2587-2599.

44 Geng, B., Zhang, C., Wang, C., Che, Y., Mu, X., Pan, J., Xu, C., Hu, S., Yang, J., Zhao, T., Xu, Y., Lv, Y., Wen, H., Liu, Z. and You, Q. (2017). IκB-kinase-ε in the tumor microenvironment is essential for the progression of gastric cancer. Oncotarget, 8(43).

45 Möser, C., Meissner, M., Laarmann, K., Olbrich, K., King-Himmelreich, T., Wolters, M., Geisslinger, G. and Niederberger, E. (2016). The protein kinase

IKKepsilon contributes to tumour growth and tumour pain in a melanoma model. Biochemical Pharmacology, 103, pp.64-73.

46 Rajurkar, M., Dang, K., Fernandez-Barrena, M., Liu, X., Fernandez-Zapico, M., Lewis, B. and Mao, J. (2017). IKBKE Is Required during KRAS-Induced Pancreatic Tumorigenesis. Cancer Research, 77(2), pp.320-329.

47 Péant, B., Forest, V., Trudeau, V., Latour, M., Mes-Masson, A. and Saad, F. (2011). IκB-Kinase-ε (IKKε/IKKi/IκBKε) expression and localization in prostate cancer tissues. The Prostate, 71(10), pp.1131-1138.

48 Hildebrandt, M., Tan, W., Tamboli, P., Huang, M., Ye, Y., Lin, J., Lee, J., Wood, C. and Wu, X. (2012). Kinome expression profiling identifies IKBKE as a predictor of overall survival in clear cell renal cell carcinoma patients. Carcinogenesis, 33(4), pp.799-803.

49 Challa, S., Guo, J., Ding, X., Xu, C., Li, Y., Kim, D., Smith, M., Cress, D., Coppola, D., Haura, E. and Cheng, J. (2016). IKBKE Is a Substrate of EGFR and a Therapeutic Target in Non-Small Cell Lung Cancer with Activating Mutations of EGFR. Cancer Research, 76(15), pp.4418-4429.

50 Chaffer, C.L., San Juan, B.P., Lim, E. et al. Cancer Metastasis Rev (2016) 35: 645. https://doi.org/10.1007/s10555-016-9648-7