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LOYOLA UNIVERSITY CHICAGO

THE ROLE OF C-ABL KINASE IN HCC DEVELOPMENT

A THESIS SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

MASTER OF SCIENCE

PROGRAM IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

BY

LENNOX CHITSIKE

CHICAGO, ILLINOIS

AUGUST, 2016

I dedicate this thesis to my mother

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iv

LIST OF FIGURES vii

ABSTRACT viii

CHAPTER 1: INTRODUCTION

1.1 HCC incidence, mortality and clinical management 1
1.1.2 HCC etiology and progression 3
1.1.3 Sorafenib as systemic therapy drug for HCC 5
1.2 c-Abl kinase 6
1.2.1 c-Abl structure and function 6
1.2.2 c-Abl Regulation 9
1.2.3 c-Abl activation in cancer 10
1.3 Axl 12
1.3.1 Axl and its role in cancer 12
1.3.2Axl activation and functional cascades 15
1.4 Project Rationale and Aims 15
1.4.1Rationale and Research Strategy 15
1.4.2 Hypothesis 18
1:4.3 AIMS 18

CHAPTER 2: INHIBITING C-ABL EXPRESSION IN HUH7 CELL LINE ATTENUATES CELL GROWTH, INVASION, MIGRATION AND THESE PROCESSES SEEM TO BE MEDIATED THROUGH AXL 19
2.1 INTRODUCTION 19
2.1.1Materials and Methods 20
2.3 Results 24
2.3.1 c-Abl is necessary for cell growth and proliferation in vitro 24
2.3.2 c-Abl promotes cell migration in HCC 25
2.3.3 c-Abl promotes cell invasion in HCC 25
2.3.4 c-Abl promotes cell growth and proliferation in xenograft in vivo mouse model 27
2.3.5 c-Abl function is partially mediated by Axl 28
2.4 Discussion and Conclusions 29

CHAPTER 3: Inhibiting c-Abl activation attenuates tumor cell growth and proliferation in vivo 30
3.1 Introduction 30
3.2 Materials and Methods 31
3.3 Results 33
3.3.1 c-Abl activity is necessary for cell growth and proliferation in vivo 33
3.4 Discussion and Conclusions 34

CHAPTER 4: c-Abl knockdown has synergistic effects with Sorafenib in inhibiting HCC growth in vitro and in vivo 36
4.1 Introduction 36
4.2 Materials and Methods 37
4.3 Results 37
4.31 c-Abl knockdown enhances Sorafenib growth inhibition and apoptosis induction in vitro 37
4.3.2 c-Abl knockdown enhances Sorafenib-mediated HCC cell growth inhibition in vivo 39
4.3.3 Sensitization of c-Abl KD cells to Sorafenib treatment occur through a c-Abl-Axl axis 42
4.4 Discussion and Conclusions 44

BIBLIOGRAPHY 47

VITA **51**

LIST OF FIGURES

Figure 1: EASL staging and clinical practice guidelines management of hepatocellular-carcinoma 2

Figure 2: Schematic of environmental, genetic etiological factors and molecular changes that occur during HCC development 4

Figure 3: Modular structure of c-Abl 6

Figure 4: Chemical structures of Imatinib, Nilotinib, Dasatinib and Sorafenib 8

Figure 5: Schematic representation of downstream events upon binding of Gas6 to Axl in different cells 14

Figure 6: c-Abl overexpression and hyperactivation is associated with poor clinical prognosis 17

Figure 7: c-Abl and Axl shRNA sequences 20

Figure 8: c-Abl promotes cell growth and proliferation in HCC in vitro 24

Figure 9: c-Abl promotes cell invasion and migration in HCC in vitro 26

Figure 10: c-Abl promotes cell growth and proliferation in HCC in xenograft mouse model 27

Figure 11: Axl is the major target and mediator of c-Abl in HCC huh7 cell line 29

Figure 12: Met/Cat HCC liver cancer mouse model scheme 32

Figure 13: Activated c-Abl promotes cell growth and proliferation in HCC in vivo 34

Figure 14: c-Abl inhibition sensitizes HCC cells to Sorafenib treatment and Sorafenib-induced apoptosis in vitro 39

Figure 15: c-Abl inhibition sensitizes HCC cells to Sorafenib treatment in vivo 41

Figure 16: Sensitization of c-Abl KD cells to Sorafenib treatment occur through c-Abl-Axl axis 43

Abstract of Thesis

Hepatocellular Carcinoma (HCC) is the second most lethal cancer after pancreatic cancer, killing almost a million people worldwide every year. Unresectable HCC tumors (intermediate to advanced stage) carry a poor prognosis and very few treatment options are available. The dismal prognosis is mainly due to limited therapy options with Sorafenib as the only approved FDA-approved drug to treat HCC. Molecularly targeted therapy against important HCC oncogenic drivers has been proposed as solution to the problem. Here, we report a novel role of c-Abl in HCC development. We provide evidence of c-Abl activation in human HCC samples compared to normal human liver samples. Using complimentary genetic and pharmacological tools, we show that c-Abl plays a vital role in promoting cell growth, proliferation, migration and invasion *in vitro* and tumor growth *in vivo*. We have identified Axl as a possible effector in these processes mediated by c-Abl. Our findings also show that c-Abl inhibition has synergistic effects with Sorafenib treatment and that this synergism occurs because Sorafenib induces activation of both c-Abl and Axl and their downstream targets. Taken together, these data we have thus far provide evidence for c-Abl as a potential critical oncogenic driver that maybe a viable target for HCC therapy, either alone or in combination with Sorafenib.

Key words: c-Abl, proliferation, migration, invasion, chemosensitization

Chapter 1:

INTRODUCTION

1.1 HCC incidence, mortality and clinical management

Hepatocellular carcinoma (HCC) is the most aggressive form of liver cancer, comprising 83% of all liver cancer cases. Globally, it is the 5th commonest malignancy in the world and is second only to lung cancer in total cancer-related deaths annually. Even though it used to be confined mainly to developing countries, which made it less of a priority in the scientific community, HCC incidence has been on the rise in Europe and the US in the past decades, with the rates more than doubling in the US. What makes the case for HCC even more pressing is the fact that it is the second most lethal cancers (8.9% 5 year survival in the US) after renal cell carcinoma (4.4% 5 year survival). [1, 2]

Surgical resection, liver transplantation and ablation are the only curative therapies available but they are only available for 30% of HCC patients with very early and early stage HCC (Figure1). 80% of HCC cases are presented late because early stage liver is asymptomatic and no reliable biomarkers are available to identify patients with incipient HCC. For those already with the disease imaging and AFP, a biomarker in about 50-70% of patients, are usually used for diagnosis. Unfortunately, patients who undergo tumor resection tend to be also associated with recurrence (50% in 2 years) and for some patients eligible for living donor liver transplants, the wait time can be long and die before a matching donor is found.

For HCC patients diagnosed with intermediate stage HCC and functional livers, transcatheter arterial chemoembolization (TACE) is their only option. TACE involves injection of cytotoxic drugs into the

arteries that feed tumor vasculature. In cases where intermediate HCC patients have dysfunctional liver or HCC is in advanced stage, Sorafenib becomes the only option (Figure 1).[3-8]

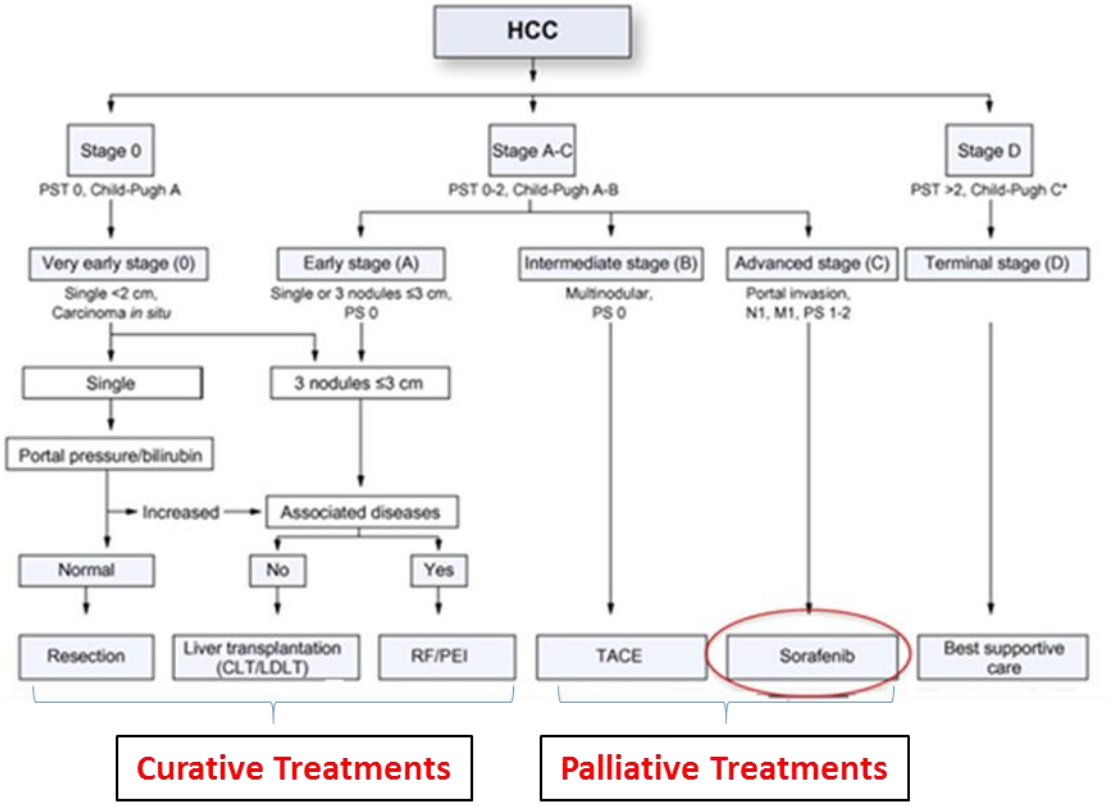

Figure 1. EASL staging and clinical practice guidelines management of hepatocellular-carcinoma. Sorafenib (circled in red) is the only FDA-approved drug for HCC. (Adapted from Forner. A et al).[3]

1.1.2 HCC etiology and progression

HCC is a very complex disease whose etiologies originate from chronic HBV and HCV infections, chronic alcohol consumption, aflatoxin and various other causes of liver injury that result in liver cirrhosis and excessive accumulation of collagen. HCC then progresses due to changes in a number of genetic and epigenetic profiles that affect key proto-oncogenic pathways. Genomic integration of HBV can lead to HCC in a number of ways, including microdeletion of genes that are related to cancer or inactivation of tumor suppressor genes. Alternatively, the Hbx viral protein can alter the transcriptional profiles of genes related to cell growth. HCV can also induce HCC in a similar fashion by inactivating tumor suppressor genes, have its core proteins interact with effectors of the cell's standard proliferative pathways or causing ER stress. Alcohol leads to HCC mainly by inducing liver cirrhosis. Alcohol creates a pro-inflammatory environment with excess cytokines and chemokines such as TNFα, IL1-β, and IL-6. This environment will lead to death of hepatocytes in the short-term which induces a cycle of regeneration and death of cells (Figure2). Eventually this cycle leads to stellate activation and cirrhosis, a major risk factor of HCC.[1, 3-4, 8] Aflatoxin is a mutagenic fungi toxin found in stored foods such as nuts, grains, and spices. The mutagenic effects of this toxin have been shown to mutate TP53 and HRAS.

Ultimately, the aforementioned etiological factors ultimately will not work in isolation in driving HCC development. For example HBV, HCV, aflatoxin are all known to inactivate p53 during pathogenesis of HCC. HBV, HCV and alcohol create a pro-inflammatory and oxidative stress environments or liver cirrhosis, which all can accelerate the chance of developing HCC. In addition to these environmental factors, HCC also has a genetic basis. Many molecular analyses have shown that certain risk factors increase genomic instability through telomere shortening and chromosomal defects. Epigenetic

aberrations in genes like COX2, E-cadherin have been shown to play a role in HCC development. β-catenin of the Wnt singling as well as Met, ErbB, PI3K, VEGFR, Hedgehog are also other key proteins that have been implicated in HCC. [1, 3, 4].

Even though these etiological factors have been identified and validated, gaps still exists in our understanding of how they can be fully exploited for the benefit of HCC patients. Identifying and validating oncogenes and biomarkers for the initiation and progression of HCC in conjunction with developing murine models that better recapitulate the genetic and micro-environmental factors observed in the clinic is therefore paramount for development of better drugs.[8]

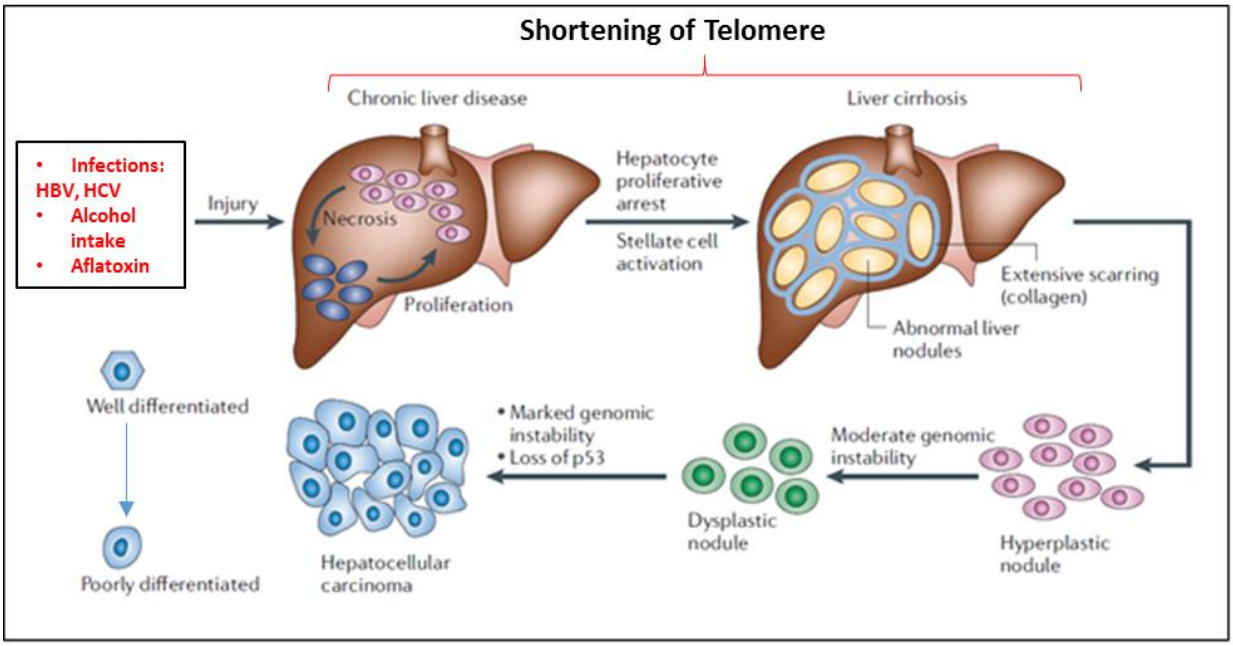

Figure 2. Diagram above shows how initial necrosis resulting from hepatic injury from various sources leads to cycle of regeneration and destruction of liver cells. These events become the driver of liver cirrhosis development, which is itself a major risk factor for HCC. (Adapted from Farazi PA et al)[1]

1.1.3 Sorafenib as systemic therapy drug for HCC

The American Association for the Study of Liver Diseases (AASLD) and European Organization for Research and Treatment of Cancer (EORTC) do not recommend use of cytotoxic chemotherapy due to marginal therapeutic benefit for HCC patients. HCC is known to be an intrinsically resistant cancer to therapy and patients usually have dysfunctional livers for systemic drug administration resulting in low response rate and overall survival. The only drug approved for advanced stage HCC is Sorafenib (Nexavar) and it is a molecularly targeted drug (Figure 4). Sorafenib has been shown to inhibit the autophosphorylation of key receptor tyrosine kinases (RTKs), VEGFR (1,2,3), PDGFR (α, β), FGFR, c-KIT, Ret, and isoforms of serine/threonine kinase Raf (1 and β). Because HCC is a highly vascularized cancer, [9-11] Sorafenib's effectiveness against HCC stem from ability not only to slow down growth but also to inhibit the angiogenic phenotype of the tumor stroma via blockade of VEGFR and PDGFR of endothelial cells. With time however, Sorafenib's effectives wanes as HCC acquires resistance with continued treatment. Therefore, there is need for more specific key targets in HCC that can be targeted individually as well as combination treatment targets that help HCC to switch addiction after chronic treatment with Sorafenib. These combination treatment targets may be responsible for activating pro-survival and pro-growth pathways that include PI3K/Akt, JAK-Stat, oxidative stress, Endothelial to Mesenchymal Transition (EMT) pathways etc. The PI3K pathway mainly activates Akt but considering the cross-talk between PI3K and MAPK pathways, Erk has also shown to be activated in some cases. Inhibition of Akt using shRNA or pharmacological inhibitors has been shown to rescue Sorafenib resistance. Sorafenib treatment is also implicated in hypoxia-inducible pathways because it starves the tumor of oxygen through its anti-angiogenic effects. This in turn causes upregulation of hypoxia-inducible factor (HIFα). In RCC, the HIFα is a prime modulator of Axl and MET activation and expression following Sorafenib treatment. Resistance to Sorafenib does not just induce growth and survival of HCC tumor cells, it also causes an aggressive metastatic phenotype to develop, partly through up regulation

of the EMT target genes. In HCC, Axl again has been shown to be a key modulator of EMT gene expression. Upregulation of EMT genes increase migratory, invasive, metastatic abilities of HCC cells. [11-14]

1.2 c-Abl kinase

1.2.1 c-Abl structure and function

Abl1 (c-Abl from here on) is a non-receptor tyrosine kinase of the Abelson (Abl) family. It is mostly known for its involvement in the so called Philadelphia chromosomes which result from the translocations of the short arms of chromosomes 19 and 22, giving fusions of the *BCR* gene to the second exon of the *C-ABL* gene and in other cases to the *ETV6* gene forming the Tel-Abl fusion. However, c-Abl was first actually discovered as a cellular homolog of v-Abl oncoprotein of Ableson murine virus and then later in blood cancers as a Bcr-Abl fusion. In both cases, the viral and fusion forms of c-Abl are constitutively active oncoproteins that transform blood cells to human leukemias, chronic myelogenous leukemia (CML) and acute lymphotic leukemia (B-ALL). [15-18]

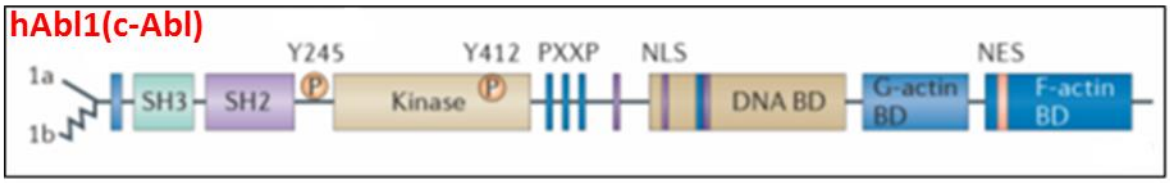

Figure 3. Modular structure of human c-Abl showing two alternatively spliced isoforms, 1a and 1b. Isoform 1b is myrostylated and 1a is not. C-Abl comprise SH domains 1, 2, 3 on the N-terminus interspersed by linkers. On the C-terminus, c-Abl has a DNA binding domain, G-actin binding domain and F-actin binding domain. The C-terminus also contains motifs: PXXP, NLS, NES. (Adapted from Grueber EK et al). [17]

In normal cells, c-Abl and its only mammalian paralogue Arg (Abl2) are involved in several cellular processes, with some functions overlapping and some distinctly different. These processes include but are not limited to, cell proliferation, cell survival, cell adhesion, cell migration, cell invasion, DNA repair pathways, cell polarity; processes that span virtually the tumor progression spectrum from initiation to metastasis when these process become deregulated. In development, c-Abl whole body knockout in mice is lethal whilst Arg whole body knockout mice are viable with minor complications, indicating that c-Abl plays an essential role in development during embryogenesis. Specifically, about three quarters of *Abl-/-* mice die postpartum and the remaining 25% that survive exhibit defects such as thymic atrophy, lymphopenia, bone mass loss, defective eye development and reduced fertility.[19] A whole body double knockout of c-abl and Arg results in mice dying early (E9-E11) in development from compromised neurological development. Studies investigating the dependency of certain phenotypes *in vivo*, can be done however by conditionally knocking out c-Abl in the organ of interest. Another salient distinction between c-Abl and Arg, in addition to their roles in development, is their subcellular localization. c-Abl is localized both to the nucleus and cytoplasm whilst Arg is localized only to the cytoplasm.[15-19] This difference localization partly explains some of the observed in differences in function between c-Abl and Arg.

Structurally, c-Abl is very similar to Src, another receptor tyrosine kinase, especially on the N-terminus (Figure 3). Like Src, c-Abl has a number of modular domains on the N-terminus: SRC homology domains SH3, SH2 and SH1 (tyrosine kinase) that play various roles. Phosphorylation of Y412 in the activation loop and Y245 in the SH2-kinase domain linker result in the stabilization and activation of c-Abl. One of the two isoforms, 1b, has a myristol moiety on the N-terminal end that plays a role in regulation of c-Abl. Unlike Src however, c-Abl has a long unique C-terminal with more distinct domains. These important domains include the filamentous (F)-actin-binding domain, the globular (G)-actin binding domain and a DNA-binding domain (DBD). The actin-binding domains are involved in cytoskeletal

organization and localization to the actin-rich regions formed during cell extracellular remodeling and other sites found in focal adhesions and invapodia. The DBD has implications on the nuclear presence and function of c-Abl. c-Abl has been shown to bind A-T rich regions in DNA and if not bound, it has been shown to associate with key nuclear proteins to include p53, Rb, ATM, E2F-DNA binding complex, transcription factor RXF1, RNA-polymerase II carboxyl terminal domains in non-transformed cells. Most of these nuclear effectors are involved in apoptosis.[20a-b] In addition to the DBD, c-Abl has motifs on the C-terminus involved in nuclear export and import. Specifically, c-Abl has 3 nuclear localization signals (NLS) and one nuclear export signal (NES). These unique motifs give c-Abl the ability to shuttle between the nucleus and cytosol.[15] Another equally important motif is the PXXP consensus adjacent to the kinase (SH1) domain and it enables c-Abl to bind to SH3 domains of adaptor proteins like Crk/CrkL, Grb2, Nck.

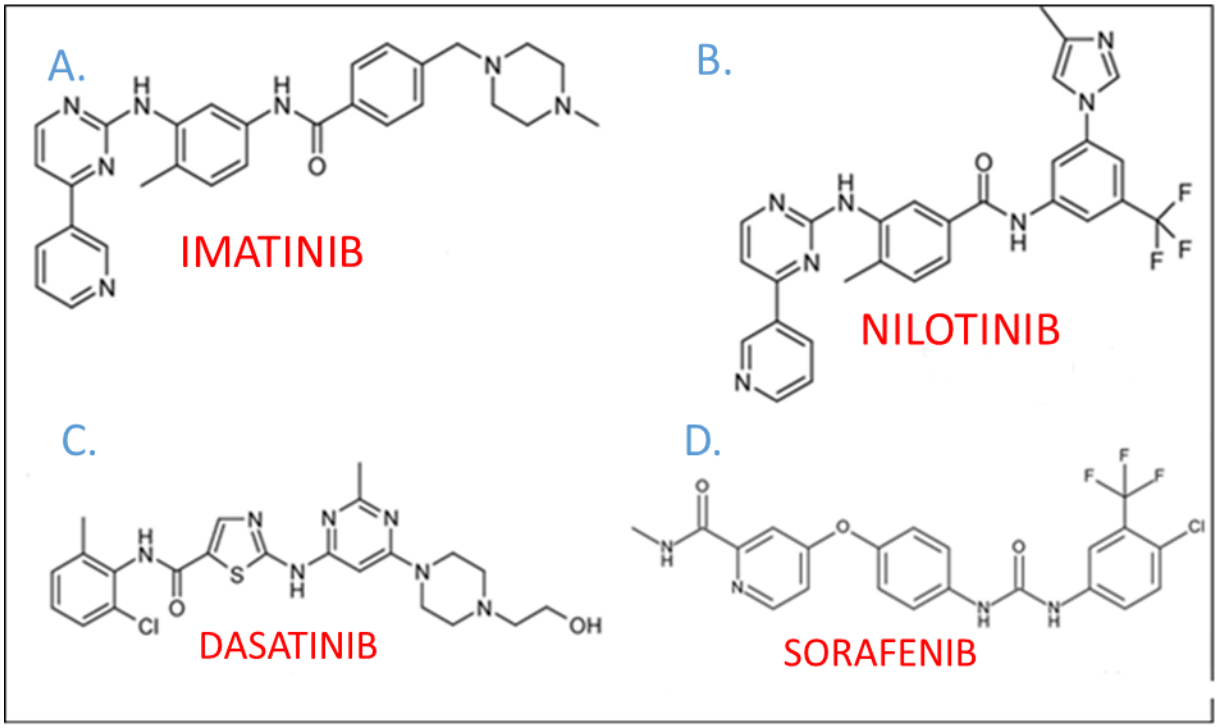

Figure 4. Chemical structures of c-Abl inhibitors: Imatinib (A), Nilotinib (B), Dasatinib (C) and HCC multikinase inhibitor, Sorafenib (D). (Adapted from Rix U et al)[42]

1.2.2 c-Abl Regulation

Under normal conditions, the function of c-Abl is tightly regulated to prevent unintended activation. c-Abl is usually in a closed conformation held in check mainly by intramolecular and intermolecular interactions. Intramolecular interactions involve binding of domains within the c-Abl itself whilst intermolecular interactions result in binding of c-Abl with other partners and cellular inhibitors in the cell. Together, these two interactions ensure that phosphorylation of key tyrosines in the mobile activation segment or loop and subsequent structural rearrangement does not happen to expose the catalytic cleft where the ATP substrate binds. The inactive conformation of c-Abl is maintained by intramolecular interactions of the SH3 domain to the linker between SH2 and the N-lobe kinase domain and the SH2 domain to the C-terminal lobe of the kinase domain. SH3 domain binding to the linker blocks the Y245 site and the SH2 domain blocks Y412 in the activation loop. Mutational studies without the SH3 and SH2 domains have been shown to increase the kinase activity of c-Abl. The myristoylated isoform 1b of c-Abl, that arises from alternative splicing, also contributes to the stabilization of the closed conformation by binding to a hydrophobic region in the kinase lobe.

As opposed to the intramolecular interactions that result in autoinhibition, intermolecular interactions can result in either inactivation or activation of c-Abl kinase activity. For example, binding partners Fus1 and peroxirodexin, have been shown to stabilize the autoinhibited state. Adaptor proteins Ras and Rab interactor 1 (RIN1), c-Cbl on the other hand result in activation of c-Abl kinase activity. Some of these adaptor proteins activate c-Abl by binding to the SH3 and SH2 domains which outcompete intramolecular interactions involved in maintaining the autoinhibited state. Adaptor proteins however only offer a partial structural relief from autoinhibition. For more activation, c-Abl has to be fully phosphorylated on certain tyrosines as they get exposed. The source of this activation could be autophosphorylation and phosphorylation of key tyrosines 245 and 412 is particularly important for

stabilizing the open conformation by preventing the open conformation from reverting back to the closed state. It is also very common for these two tyrosines to be phosphorylated in trans by other kinases such Src family kinases or receptor tyrosine kinases (e.g. PDGFR, Her2) are able to interact and phosphorylate c-Abl.[17, 21- 23, 26] With Y245 and 412 phosphorylated, c-Abl becomes maximally activated. Kinase activity resulting from activation can be measured using antibodies against Y245 or Y412 on c-Abl. Another popular way to measure c-Abl activity is using an antibodies against pCrk or pCrkL as surrogates for c-Abl kinase activity. Even though Crk and CRKL are targets of a few other kinases, they are mainly phosphorylated by c-Abl at tyrosines 221 and 207, respectively. Both Crk and CrkL physically bind to the proline rich-domain on the c-terminus of c-Abl via their SH3 domain during phosphorylation.[19, 24]

1.2.3 c-Abl activation in cancer

Even though c-Abl has been notoriously known for a long time now for its role in leukemias harboring the Philadephia chromsomes, relatively recent evidence has shown that c-Abl (and Arg) play role in progression of solid tumors by independent mechanism not involving any fusion oncoproteins. These mechanisms have mainly been pinned down to copy number amplifications, mutations and catalytic activation resulting from hyperactive receptor tyrosine kinases or growth factor signaling.[17, 25-31] Copy number amplification results in overexpression which in turn enhance the activation of Abl kinases. Copy number amplifications may then lead to an increase in the expression of c-Abl at DNA level. A number of immunohistochemistry studies have demonstrated that c-Abl expression is upregulated in a number of cancers to include brain, lung, ovarian, colon cancers etc. Indeed, many studies have proposed that c-Abl oncogenic function is often a result of activation via integrins, hyperactive adhesive receptors or mitogenic growth factor signaling. Using a variety of methods, activity of c-Abl has been correlated with receptor kinases that play important roles in cancer. Some of the main receptors that have been identified as activating to c-Abl include ErbB (EGFR), PDGFR, IGF-1R and c-Met, $\alpha_v\beta_3$.

Downstream of c-Abl are a number of interacting proteins to include Stats, Erk, cortactin, CrkL, paxillin, Fak etc. Together with these interacting partners, c-Abl has already been shown to transduce signals that control cell proliferation, survival, cell migration, invasion, adhesion, EMT, metastasis and even chemo-resistance in different cancer cell lines[15, 25-31] Specifically, Abl kinases have been shown to control growth through c-Myc, JNK, Erk and Stats[25, 31-33] Cell migration, invasion, and EMT have been shown to be mediated through Rac, cortactin, metalloproteinases and β-catenin.[25, 34-36] In prostate cancer, c-Abl activation via PDGF led to increased survival by upregulating MCL-1, an anti-apoptotic protein, and signaling the p68/β-catenin pathway. c-Abl has also been shown to play a role in drug resistance via activation by Axl. [37] There are several methods that have been employed to show the significance of c-Abl activation in solid tumors. RNAi and shRNA genetic tools have been used in conjuction with c-Abl inhibitors to show the dependency of phenotypes of many solid tumors. [33, 38-41]. They are several Abl inhibitors currently available in the clinic for the treatment of Abl-positive luekemias, particularly CML. These mainly include imatinib (Gleevec/STI-570), Nilotinib, Dasatinib and Ponatinib (Figure 4). Imatinib is the frontline drug for CML patients at the moment. It blocks the binding of ATP to the kinase domain of Abl as well as other kinases like c-Kit and PDGFR albeit to a lesser extent. With time however, CML patients develop resistance either through increased expression of BCR-Abl or mutations that compromise the binding of Imatinib. To overcome this resistance and imatinib intolerance Nilotinib, Dasatinib and Ponatinib have been developed as second-generation drugs. Ponatinib is the only drug that has been shown thus far to effectively to rescue the so-called T315I mutation in which a threonine to isoleucine substitution in the kinase domain of Abl prevents binding of imatinib. Nilotinib is a close analog of imatinib that binds to the inactive conformation of the kinase domain of Abl with better selectivity and higher potency than imatinib. It has been to be approximately 20 times more potent than imatinib with even more selectivity towards Bcr-abl and c-Abl. Dasatinib is a multi-kinase inhibitor of Bcr-Abl and Scr family kinases. It has been found to be even more potent than Nilotinib or Imatinib but

because it targets about 30 other kinases. Some of these other kinases are key effectors in the immune system and not surprisingly, it causes cytopenia and pleural effusions.[42, 43] In solid tumors, imatinib and nilotinib have mainly been used to study consequences of inhibiting Abl activation. Some of the well-studied cases of c-Abl activation were done in cell lines derived from breast, colon, lung and kidney carcinomas, and melanoma.

1.3 Axl

1.3.1 Axl and its role in cancer

Axl is one of the RTKs in the TAM (TYRO3, AXL, MER) receptor family. It is a multi-domain receptor comprising 3 domains: extracellular, transmembrane and intracellular kinase domains. The extracellular domain consists of two immunoglobin(Ig)-like domains and two fibronectin-type III repeats. The transmembrane domain links the extracellular to the intracellular domain on the C-terminal lobe. The kinase domain is responsible for activation of receptor. Axl has been implicated in a number of cancers either through overexpression or over activation. In most tumors, Axl is usually not a strong primary oncogenic driver itself but rather an avenue for cells to re-gain survival advantage when the tumor cell is under stress. In line with this premise, Axl has been found to promote survival, chemoresistance, aggressive metastatic phenotype through facilitation of cell migration, motility, invasion and Epithelial to Mesenchymal Transition (EMT). The number of Axl inhibitors that have been approved or currently being developed in the clinic underscores the importance of Axl as a primary or secondary target.

Activation of Axl happens through ligand-dependent (Axl/GAS6 axis) or ligand-independent mechanisms (Figure 5). Ligand-dependent mechanisms is the commonest and it involves two AXL ligands called GAS6 which bind individually to the Axl extracellular domain causing Axl receptors to dimerize. This dimerization then promotes trans-autophosphorylation of the receptors. In cancers, activation GAS6 can happen in an autocrine or paracrine fashion. Additional mechanisms of activation which do not include a

ligand also exist. Under oxidative stress, homophilic dimerization can occur resulting in activation. Axl overexpression can also promote homophilic dimerization and this is a common occurrence. Axl has been found to be overexpressed in a number of cancers to include renal cell carcinoma (RCC), hepatocellular carcinoma, breast cancer etc. Overexpression of Axl in most cases has been found to be associated with dismal patient prognosis. Overexpression of Axl molecules often leads to autophosphorylation even in the absence of GAS6. Activation can also occur via heterophilic dimerization where Axl dimerizes with a different RTK and result in activation signaling. This could be with another TAM RTK as with Tyro3 or with an RTK from a different family such as FLT3 during NK cell development.[44-46]

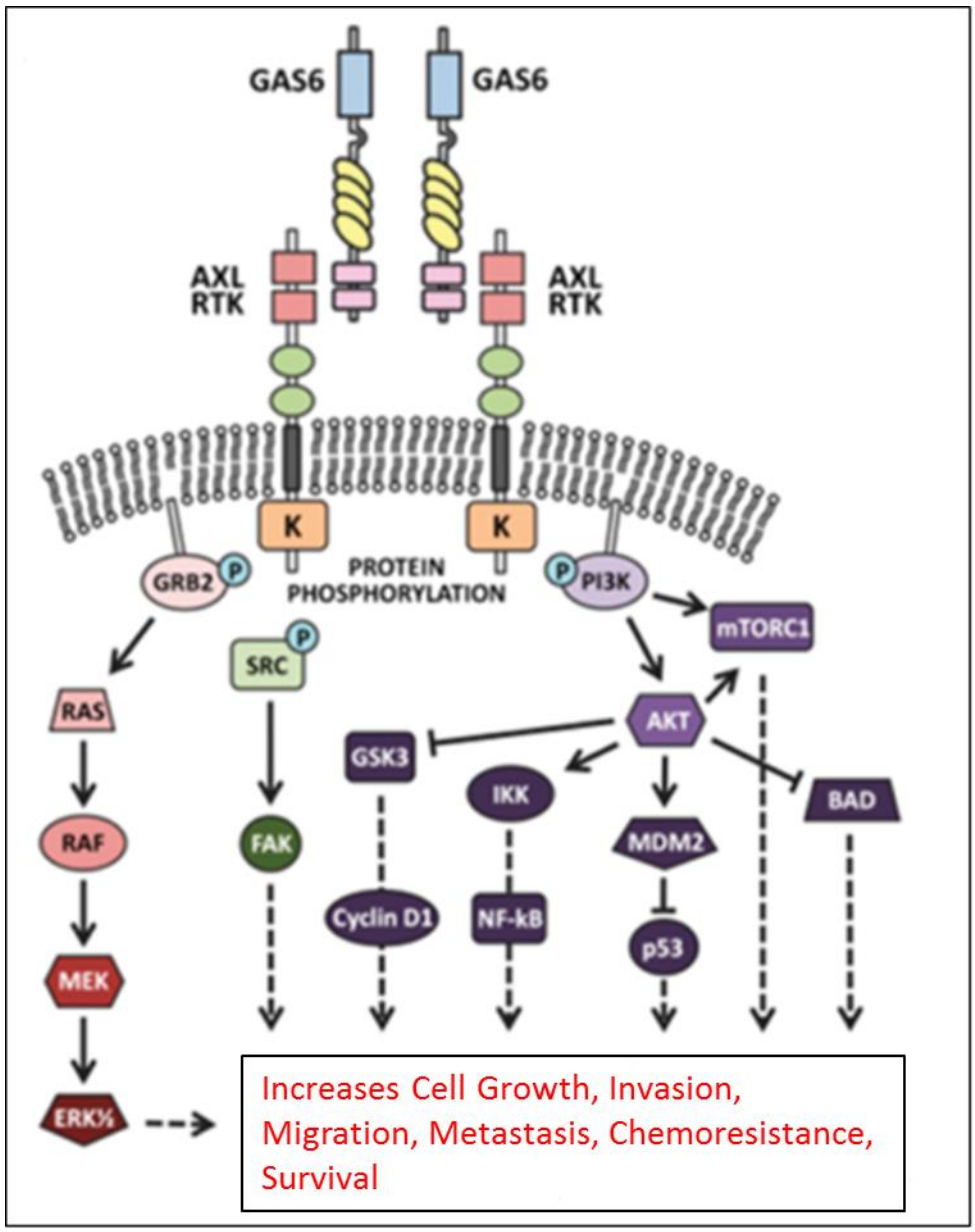

Figure 5. Schematic representation of downstream events upon binding of Gas6 to Axl in different cells and contexts. Axl domains Ig-like 1 and Ig-like 2 make contact with the LG1 domain of Gas6 causing dimerization and autophosphorylation and Axl activation, which elicits many different process important in cancer progression. (Adpated from Myers SH et al).[44]

1.3.2Axl activation and functional cascades.

In tumor cells Axl can get activated through various mechanisms described above. As alluded to already, Axl activation plays a considerably important role when tumor cells is subjected to stress as well. Stress could result from oxidative stress, hypoxia or chemotherapy drugs. These stressful conditions will lead to dimerization and subsequent autophosphorylation of tyrosine residues on the kinase domain, especially Y779, Y821 and Y866 which leads to signaling of proliferative, survival or EMT pathways. Specifically, Axl has been shown to activate the Ras/Raf/Mek/Erk pathway that promotes proliferation and PI3K/Akt/MTOC1, NFkB or Bcl-2 pathways which are all pro-survival. Axl also promotes induction of EMT-promoting genes such as SNAIL. Prime examples that demonstrate these phenomena include findings of Axl heterodimerizing with EGFR or PDGFR in breast chemoresistance. Axl has also been shown to cross-talk with MET and Src in both hypoxic and chemotherapy conditions in RCC and breast cancer, respectively. In both cases, stress results in activation of Axl, which compensates by upregulating pro-survival pathways. In esophageal cancer, a similar counter measure to chemotherapy involving Axl-Abl1 axis was shown to rescue cancer cells from the cytotoxic effects of drugs. In all these circumstances involving chronic treatment with a drug, cancer cells, with the help of Axl, are able to resurge with a more aggressive phenotype. [47-50]

1.4 Project Rationale and Aims

1.4.1Rationale and Research Strategy

Hepatocellular Carcinoma (HCC) is the second most lethal cancer after pancreatic cancer, killing almost a million people worldwide every year.[1,2] Unresectable HCC tumors (intermediate to advanced stage) carry a poor prognosis and very few treatment options are available. The dismal prognosis is mainly due to a lack of therapy options. Despite a relatively recent increase in interest in HCC from the Western scientific community, Sorafenib is the only systemic therapy option thus far; cytototixic agents do not

give any appreciable survival advantage and are practically not in use. Sorafenib, the only FDA approved drug, is a multikinase inhibitor that has about a 3-month survival advantage. Despite this sense of despondency, the advent of molecularly targeted therapies and improvement in prognosis in similarly challenging cancer such as Renal Cell Carcinoma (RCC), in part due to targeted therapies, there is a ray of hope in studies that identify specific molecular pathways that drive cell growth, invasiveness or angiogenesis in HCC for rational therapy.[3,4].

We believe c-Abl, non-receptor kinase, is a novel target in HCC that deserves to be investigated for its role in HCC development and potential as a drug target. A comprehensive search in the literature has shown that c-Abl is integrated in different cytoplasmic oncogenic signaling pathways involving integrins, cytokine and growth factor receptors during tumor initiation and progression of other solid tumors. In most of these cases, c-Abl is hyperactive due to phosphorylation by upstream signaling partners. In other cases, c-Abl is overexpressed or mutated; with its mammalian paralogue Arg, overexpressed even more. [25-30] Our preliminary search in the c-BioPortal and Oncomine data sets showed that c-Abl overexpression is associated with poor clinical prognosis (Figure 1). However, we did not find evidence in the literature of oncogenic function of c-Abl in HCC. Our preliminary *in vitro* studies using Abl kinase inhibitor, Nilotinib, we found that indeed, Abl inhibition significantly slows down HCC cell growth. More importantly we found c-Abl to be hyperactivated in human HCC samples relative to normal liver controls (Figure 6).

This project therefore will encompass 3 main aims to comprehensively study how c-Abl modulates cell growth, migration, invasion and tumor growth both *in vitro* and *in vivo* and explore the possibility of c-Abl as a druggable target in HCC. Overall, the aims will be achieved by use of a complimentary experimental approach that involves genetic and pharmacological tools both *in vitro* and *in vivo*.

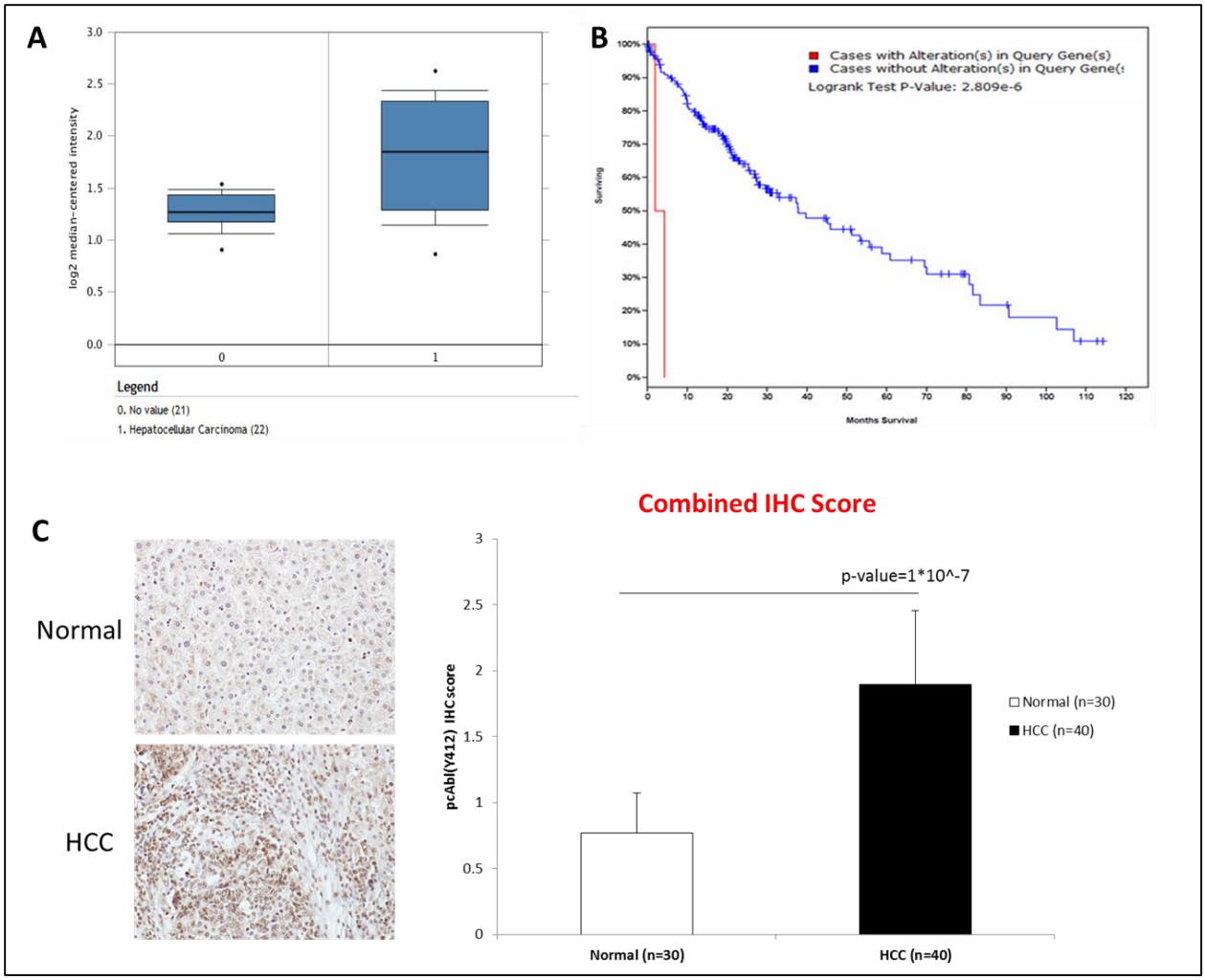

Figure 6. c-Abl overexpression and hyperactivation is associated with poor clinical prognosis. A. c-Abl mRNA expression levels in human HCC and normal human liver samples (Data compiled from TCGA/AMC data sets using Oncomine). B. Overall Survival Kaplan-Meier showing association between c-Abl DNA amplification and overall survival (left) (Data compiled from TCGA/AMC data sets using cBioPortal website). C. Activated c-Abl levels in human HCC versus normal liver samples as determined from a tissue array assay.

1.4.2 Hypothesis

Enhanced activity or overexpression of c-Abl accelerates the growth and metastatic potential of HCC cells, which drives the progression of HCC.

1:4.3 AIMS

Aim 1: Determine if c-Abl is necessary for HCC oncogenic processes *in vitro*.

- Determine whether inhibition of c-Abl expression affects cell growth, migration, invasion both *in vitro* and *ex vivo*.
- Identify and characterize c-Abl signaling partners and determine c-Abl signaling pathways in HCC development

Aim 2: **Determine if c-Abl is necessary for HCC development *in vivo*.**

- Determine whether c-Abl-specific pharmacologic inhibition using Nilotinib slows tumor cell growth *in vivo*.

Aim 3: To determine whether c-Abl is a potential viable candidate for combination therapy with Sorafenib

- Determine if c-Abl knockdown sensitize HCC cells to Sorafenib treatment in vitro and ex vivo
- Determine the mechanism of synergy between c-Abl inhibition and Sorafenib treatment in HCC.

CHAPTER 2

Inhibiting c-Abl expression in huh7 cell line attenuates cell growth, invasion, migration and these processes are partially mediated through Axl

2.1 Introduction

Several studies have demonstrated the importance of c-Abl in a number of solid tumors. Inhibiting the expression of c-Abl has been shown to reverse or slow down some of these phenotypes. In some cases, c-Abl has been to be overexpressed and this has been positively correlated with poor clinical prognosis. The cytoplasmic pool of c-Abl has been shown to be important in the signaling that drives processes that favor solid tumor progression. c-Abl drives oncogenic processes by either linking with growth factors or integrins to activate its downstream targets like CrkL, Rac, WAVE3 which usually effect processes that pertain to cell movement. In other cases however, c-Abl has been shown to crosstalk with pathways involving survival, proliferation or EMT such as PI3K, MAPK, Wnt pathways. [25-30] c-Abl has also been shown to interact directly with Axl in esophageal cancer and this interaction was shown to be key for esophageal tumor cells to mount resistance to cisplatin. The specific processes that have been shown to be affected include but not limited to, cell growth, invasion, migration, survival, and metastasis.

Here we investigate whether c-Abl is necessary for cell growth, invasion, migration and the effectors that mediates c-Abl's oncogenic function in HCC. We also provide mechanism of how these processes are elicited and provide insights into whether c-Abl can be used in drug development efforts for HCC. We show that c-Abl promotes cell growth, invasion and migration of HCC Huh7 cells both in vitro and in vivo and that Axl is involved, directly or indirectly, in cell growth, migration and invasion.

2.1.1 Materials and Methods

Cell lines and Treatments

Huh7 cells were grown and maintained in Dulbecco's modified Eagles's (DMEM) high glucose medium (Thermo Scientific, Waltham, MA) supplemented with 10% FBS, penicillin, and streptomycin (Sigma, Aldrich, St, MO) in a humidified chamber of 5% CO2 at 37^{o}C. To knockdown c-Abl expression, lentiviral pLKO.1 plasmid for c-Abl, Axl or scrambled shRNA was packaged with pCMV-dr8.2 (Addgene) and pCMV-VSVG (Addgene) in HEK 293-T cells to produce lentiviral particles. Huh7 cells were then infected with viral particles containing either c-Abl or scrambled shRNA and selected with 10ug/mL puromycin for about 7 days. Clones from the selection were then used for subsequent experiments. shRNA plasmids against c-Abl and Axl were obtained from Thermo Fisher Scientific.

Abl shRNA#2- CCGGGAGTTCTTGAAGCATTTCAAACTCGAGTTTGAAATGCTTCAAGAACTCTTTTTG
Abl shRNA#4- CCGGGCTTTGGGAAATTGCTACCTACTCGAGTAGGTAGCAATTTCCCAAAGCTTTTTG
Axl shRNA#1- ATGTTGACATAGAGGATTTCG

Figure 7. shRNA sequences used for knocking down c-Abl and Axl.

Reagents

Primary antibodies directed against GAPDH, b-actin, pErk, pAkt (S473), pSrc, pCRKL were purchased from Cell signaling Technology (Danvers, MA). Antibodies directed against Axl and pAxl (Y772) were purchased from R&D systems. c-Abl antibody was purchased from Santa Cruz , CA and pc-Abl (Y412) was obtained from Abcam. BrdU antibody was purchased from Invitrogen.

Mice and Treatment

All nude mice used in this study were treated using procedures approved by the Institutional Animal Care and Use Committee at Loyola University Chicago. Mice for xenograft experiments were first shaved under isoflourane on both right and left flanks. 5*10^^{6} huh7 cells in 100uL serum-free media were

injected into the flanks. Scrambled shRNA cells were injected to the left flank and knockdown cells injected to the right. Tumor volumes were then measured using an external calipers daily until the day of sacrifice. Specifically, the volume was calculated using the ellipsoidal formula:

Tumor Volume (mm^3)=1/2($L*W^2$) where *L* is the greatest longitudinal distance and *W* is the greatest transverse distance.[52]

Tumor volumes were monitored not to grow beyond the humane threshold of 2000mm^3 upon which tumors were harvested, weighed, stored at -80^oC or embedded for immunohistochemical analysis. The mice were housed in micro-isolator cages in a room illuminated from 7:00am to 7:00pm and allowed to access water and water ad libitum.

qPCR

RNA was collected from cells using Zymo Research Extraction Kit. DNAseI was used to remove traces of DNA. RNA concentration was quantified using Nanodrop. Reverse-transcriptase (Bio-Rad) PCR was used to create cDNA from 5 µg of extracted RNA and random primers. Target genes were then amplified using qPCR using specific primers, Syber Green kit from Bio-Rad. GAPDH primers were used internal controls. Fold change was determined using Double Delta Ct Analysis.

Cell Lysis and Western blotting

Whole cell lysates were prepared by lysing in RIPA buffer supplemented with phosphatase and protease inhibitors. Protein fractions were then run on SDS-PAGE gels using protocols from manufacturers.

Cell Growth Assay

Equal number of cells infected with scrambled and target genes shRNA were counted and seeded in 12 well plates. Pictures were taken under the microscope as soon as cells attached (~4 hours) and this was deemed time 0 hrs. More pictures were then taken at different time intervals and after images are

collected, the cells from the respective wells were then put in trypan blue and quantified using an automated cell counter.

BrdU Incorporation Proliferation Assay

Equal number of cells infected with scrambled and target genes shRNA were counted and seeded onto cover slips in a 24 well-plate. After 48 hours, the cells were stained with 0.03 mg/mL of BrdU for 30 minutes and fixed using 70% ethanol for 5 minutes. Cells were then blocked and immuno-stained with BrDu primary antibody overnight. A flourochrome-conjugated secondary antibody was then applied on cells before being visualized on a microscopy with a DAPI counterstain. A triplicate of images were taken from different fields and averaged. For BrdU stain quantification, BrdU signal was taken as a ratio of DAPI.

Matrigel Cell Invasion

Matrigel (8 mg/mL) was coated to invasion chambers for 2 hours at 37^{o}C at 100 uL and suspended in a 12-well plate. Equal numbers of cells infected with scrambled or target genes shRNA were washed in serum-free media, counted and seeded in the chambers in serum-free media. Normal media containing 10% of chemoattractant FBS was added to each well underneath the chambers. Cell were incubated at 30^{o}C in the chambers for 48 hours, with a change in the chemoattractant media at the 24-hour time point. The matrigel and cells in the upper chamber were removed with a cotton swab and only cells in the lower chamber were fixed with 10% formaldehyde and stained with H&E and quantified to assess extend of invasion.

Cell Migration Scratch Assay

Cells infected with scrambled or target genes shRNA were seeded so that they reach confluency in 24 hours. After 24 hours, a scratch was made in every well using the blunt end of a 10 uL pipette tip. Using a ruler, regions of equal width were marked with a marker pen. Pictures were taken at different

time points using the position of the marker as a guide for taking pictures in comparable regions. The rate of migration is qualitatively assessed from images under the microscope.

Paraffin-embedding and IHC

Xenograft tumor tissues were pre-fixed in formalin before dehydration in 70% ethanol. The samples were then sent to the pathology lab for embedding in paraffin blocks. After embedding, the blocks were sectioned at 5 μM on a microtome and incubated at 44^{o}C overnight. Sections were de-paraffinized in xylene and then run in 100% and 95% ethanol. Antigen was recovered by microwaving sections for 15 mins in antigen-retrieval solution. Peroxidase activity was quenched using H_2O_2 before blocking. Primary and HRP-conjugated secondary antibodies were then added before visualizing with a microscope.

RTK array phospho-proteome profiling

The cells were grown for 24 hours and harvested by washing with PBS and lysis in buffer 17 supplied by R&D systems, supplemented with a phosphatase and protease inhibitors. Cell lysates were rocked for 30 mins on a rocker and centrifuged at 10 000*g* for 5 min. RTK array membranes (R&D) were blocked in the provided blocking buffer for 1 h and incubated with 1000 μg of cell lysate overnight. The membranes were incubated with horseradish peroxidase-conjugated phosphotyrosine detection antibodies for 2 hours after washing. The signal was detected using an enhanced chemiluminescence reagent kit provided.

Statistics

Statistical analysis was carried out using a two-tailed Student t test in Excel. $P<0.05$ was considered significant. Data was presented as mean ± SD.

2.3 Results

2.3.1 c-Abl is necessary for cell growth and proliferation *in vitro*

c-Abl has been shown to play a role in many solid tumors via interactions with integrins and mitogen receptors. c-Abl, through activation by these hyperactive upstream partners, has been shown to mediate oncogenic processes from DNA synthesis to EMT in cancers like breast cancer and melanoma. We were interested in the requirement and dependency of HCC cell growth on c-Abl and we silenced c-Abl expression to study this dependency and confirmed knockdown with an immunoblot (Figure 8E).

As described in the methods section, cell growth assays were performed using scrambled and c-Abl shRNAs in huh7 cells. Our results show that cell growth was significantly lower when c-Abl was knocked down both at the 24 hr and 48 hr time points.

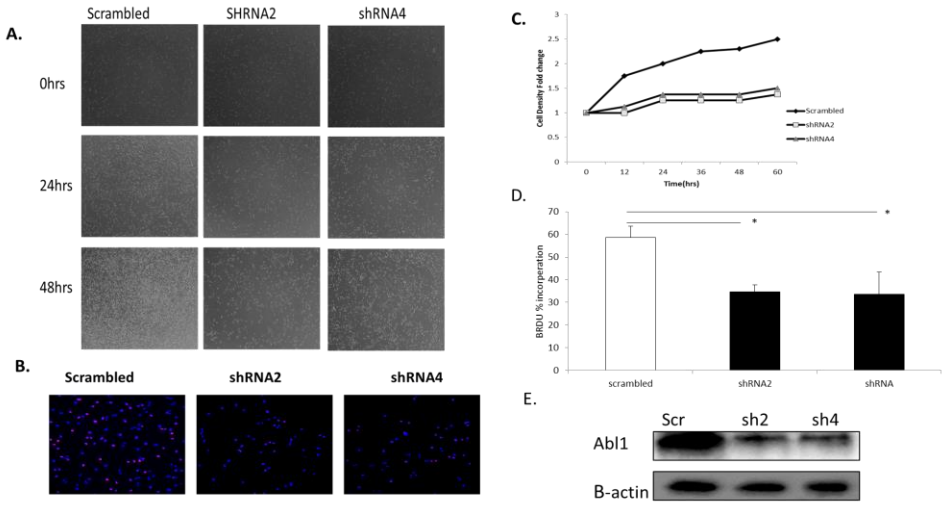

Figure 8. c-Abl promotes cell growth and proliferation in Huh7 in vitro. A,C. Cell growth assay results of scrambled shRNA and c-Abl knockdown cells from microscope images at various time points and the respective quantitative cell numbers. B,D. Cell proliferation in control and c-Abl knockdown using BrDu (pink) and DAPI counter-stain (blue) and % BrDu signal. E. c-Abl knockdown efficiency as determined by immunoblotting.

Specifically, the plate images show a clear qualitative difference, with the cells with c-Abl scrambled shRNA quicker in reaching confluency whilst the corresponding knockdown cells are still sparse. The cell numbers also show a consistent difference, with more cells in the scrambled shRNA compared to the knockdown cells. Similarly, with the BrDu incorporation assay we found that the percent (%) BrDu signal was significantly higher in the scrambled control compared to when c-Abl is silenced. These differences reached statistical significance ($p<0.05$). We confirmed the results using two different shRNa directed against c-Abl and show that c-Abl is important for cell growth and or proliferation in HCC (Figure 8).

2.3.2 c-Abl promotes cell migration in HCC

c-Abl is well known to interact with various integrins and cytoskeletal elements that modulate cell adhesion and cell migration processes via cortactin, Rac1, WAVE3, RhoA activation etc. Most of these targets have been shown to mediate Abl function in cancer.

To answer the question whether c-Abl drives cell migration specifically in HCC, we performed a scratch assay using scrambled shRNA and knockdown cells. As shown in Figure 8, we found that silencing c-Abl greatly decreased migration of cells across the scratch compared to the scrambled cells in 48 hrs. In the 48 hrs of the study, cells did little migration in knock down samples yet scrambled cells almost reached confluency in just 24hrs. These observations suggests that c-Abl is vital during Huh7 cell migration.

2.3.3 c-Abl promotes cell invasion in HCC

c-Abl has been shown to be localized to actin-rich regions known as invapodia that are formed in some solid tumors during cell invasion. c-Abl has also been shown to induce upregulation expression of proteins important for metastasis like metalloproteinases and EMT target proteins. It was of interest to know the status of c-Abl in relation to cell invasion in HCC. To this end, a cell invasion assay was conducted with matrigel as the matrix and cells were stained with H&E after 48 hours. Images were taken then taken under the microscope. The H&E signal in knock down cells is significantly weaker

compared to scrambled cells. The H&E signal is higher by at least a factor of 2 in control versus knock down cells (Figure 8). These findings show that silencing c-Abl attenuates the invasive properties of cells compared to control, indicating that c-Abl is required for cell invasion in huh7 cell line.

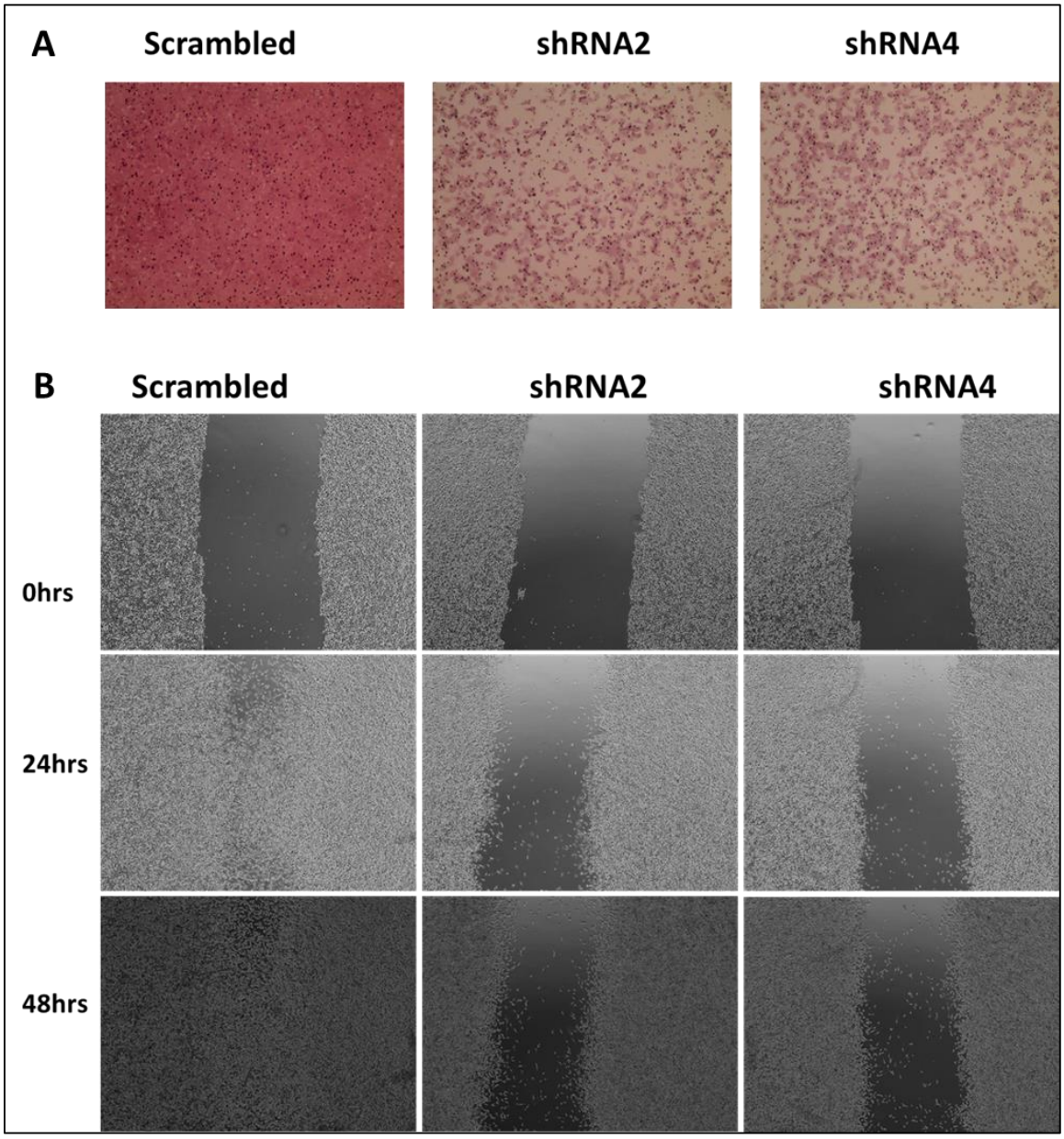

Figure 9. c-Abl promotes cell invasion and migration in HCC in vitro. A. H&E stained control and c-Abl knockdown cells from Matrigel cell invasion assay after 48 hours. C. Cell migration in control and c-Abl knockdown using a scratch assay at different time points.

2.3.4 c-Abl promotes cell growth and proliferation in xenograft *in vivo* mouse model

To test the role of c-Abl in HCC growth in vivo, a SCID-bg xenograft mouse model was used. Equal number of knockdown and control cells (5*10^^{6} huh7 cells) were injected into the right and left flanks of the SCID-bg mice, respectively and allowed to grow for about 7 days before measurements of tumor volumes were taken after every 24 hours for another week. Tumor weights from knock down cells were statistically smaller than those of control cells ($p<0.05$). Tumor volumes were also smaller in knockdown cells. In the first 3 days of measurement the differences were small (Figure 9) but in the next 4 days differences became significant ($p<0.05$). ki67 staining also show more proliferation in control versus knocked cells ($p=0.02$). Our results show that c-Abl is necessary for maximal growth *in vivo* and supports the phenotype of diminished growth observed *in vitro* when c-Abl is knocked down.

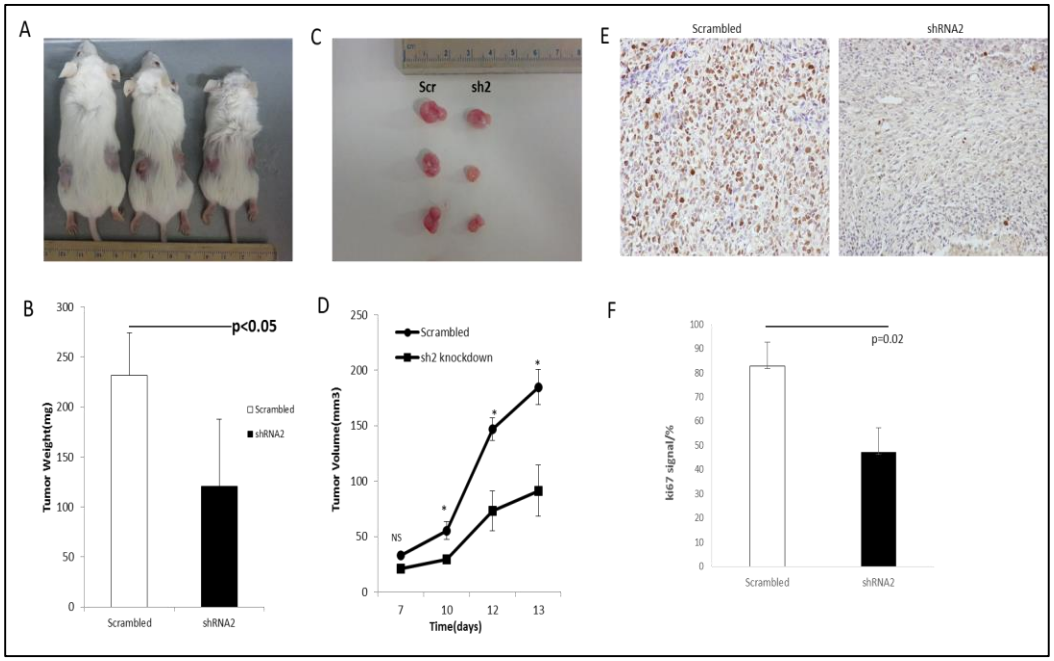

Figure 10. c-Abl promotes cell growth and proliferation in Huh7 in vivo. A, B. SCID-bg mice injected on the flanks with control (left) and knockdown cells (right) and quantitation of tumor weights ($p<0.05$). C,D. Gross images of tumors after harvest control (left) and knockdown cells (right) and tumor volumes. E,F. Cell proliferation staining in tumors using ki67 of control (left) and knockdown cells (right) and ki67 % signal ($p=0.02$). NS=not significant; *=$p<0.05$

2.3.5 c-Abl function is partially mediated by Axl

c-Abl function has been shown to be complex and context dependent. From the evidence in the literature, c-Abl has many different signaling pathways depending on the cancer type or subtype. Since there is no precedent mechanistic study of c-Abl in HCC to our knowledge, our goal was to identify and characterize c-Abl signaling partner(s) in HCC development. To this end, we sought to identify receptor tyrosine kinase (RTK) partners using an efficient non-biased approach that samples the activation status of many candidates at the same time. RTKs are some of the common targets being investigated for possible molecularly targeted therapy and identifying one that might be working with c-Abl directly or indirectly, will make the case for the c-Abl signaling pathway stronger as a target.

Specifically, with an RTK array we quantitatively profiled and analyzed the receptor tyrosine kinase phosphorylation status in scrambled and knockdown huh7 cells to deduce the effect of c-Abl inhibition on activation of certain RTKs. Our results show that c-Abl inhibition decreases phosphorylation of EphA2, 7 and Alk in HCC huh7 cell line. The other RTKs had their phosphorylation status minimally affected when c-Abl was knocked down. The RTK whose activation is greatly reduced however was Axl. The phosphorylation signal had greatest decrease relative to all the RTKs tested in this array. We followed up observation by confirming Axl expression and activation in scrambled and knock down cells with immunoblotting. Western blots showed a similar decrease in phosphorylation of Axl, with no appreciable effect on Axl expression levels (Figure11). To determine if Axl affects some of the processes that were affected by c-Abl, we also created an Axl knockdown cell line and performed cell growth, migration and invasion assays. The attenuation in growth, migration and invasion with Axl knockdown is what we observed with c-Abl knock down (Figure 11). Knockdown of Axl also decrease activation of downstream targets such as Akt, which was also significantly decreased when in c-Abl knockdown cells. This similarity adds some credence that Axl may be the effector that mediates c-Abl function in HCC.

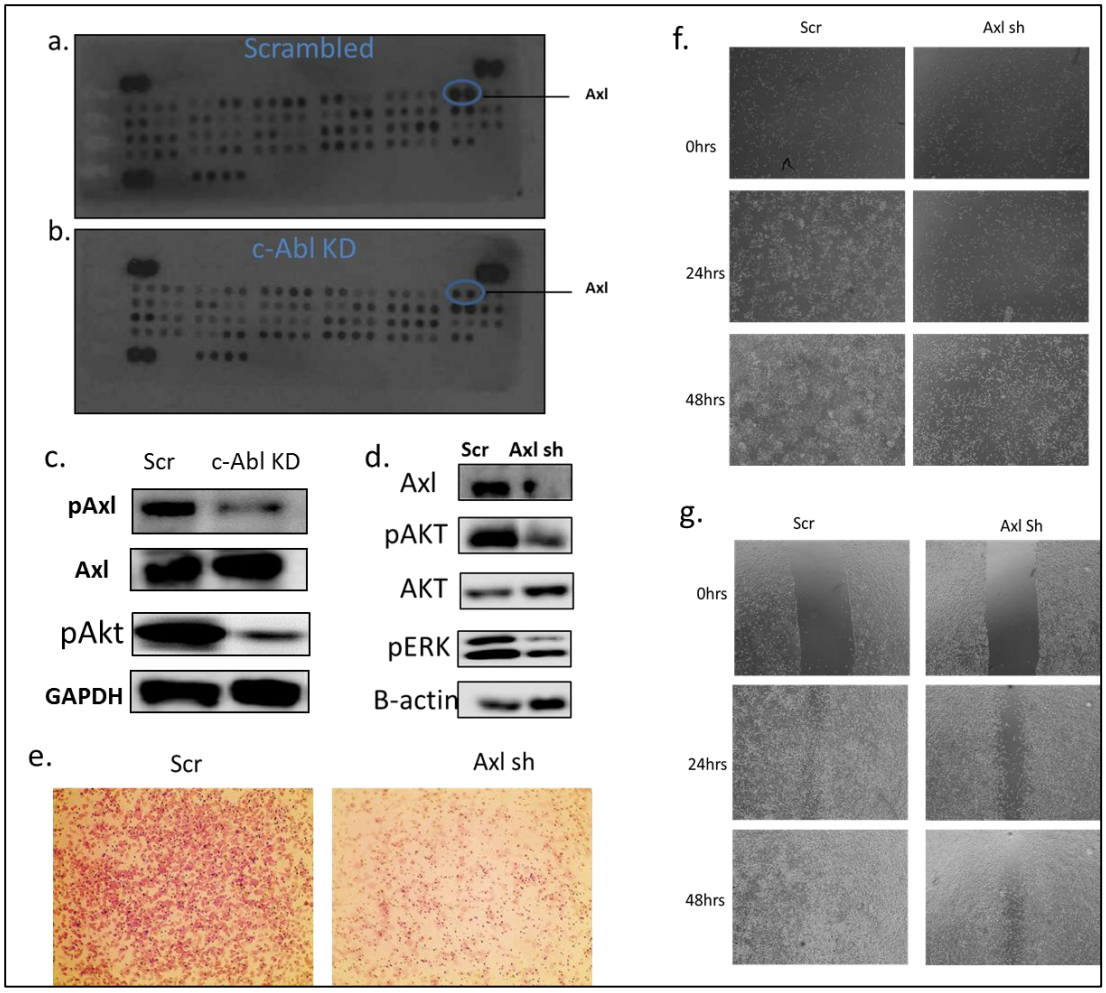

Figure 11. Axl is the major target and probable mediator of c-Abl in HCC huh cell line. a, b. Axl activation decreased the most upon c-Abl knockdown as determined by RTK array assay (a-Scrambled cells, b-knockdown cells). c,d. Immunoblot analysis confirms decrease in Axl phosphorylation upon c-Abl knockdown and decrease in pAkt, pErk upon Axl knockdown in huh7 cells. e,f, g. Axl gene silencing attenuates invasion, growth and invasion of huh7 cells, respectively.

2.4 Discussion and Conclusions

According to GLOBOCAN estimates, there were about 748, 000 new HCC cases and 695,900 deaths worldwide, a whopping 93% mortality rate.[2] A closer look at the numbers, reveals that number of people who die every year is almost identical to number of people diagnosed, which alludes to the aggressive nature of this disease.[2] This also underscores the current deficiency in biomarkers and targets for effective treatment of HCC.

In this study we reported a novel role of c-Abl in HCC development. We showed that c-Abl in critical for HCC cell growth and proliferation. Furthermore, we also showed that c-Abl is vital for cell migration and invasion. c-Abl has been demonstrated to drive these processes in a number of solid tumors via various pathways and effectors such as Erk, c-Myc, PCNA, RonA etc.[25-30] c-Abl also plays a vital role in the invapodia of melanomas and breast cancer where it affects metastastic potential by changing expression of metalloproteinases and EMT target genes. In here, we have identified a possible target that might affect processes like growth, invasion in HCC progression and this target is Axl. We confirmed the decrease in Axl activation in RTK assay by western blot. We also showed dependency of growth, invasion, migration on Axl, phenotypes that also depended on c-Abl. Indeed, Axl has been shown to be important for growth, invasion and migration in many cancers.[44-50] In HCC, Axl is usually overexpressed and has been shown to drive migration and invasion via alteration of EMT target genes. Even though, there is no study thus far that shows that there is interaction between Axl and c-Abl in HCC, c-Abl was shown to interact with Axl in esophageal cancer.[37] Further protein-protein interaction studies need to be performed to establish if there is a physical association between c-Abl and Axl in HCC or if there is a non-physical cross-talk. Future studies may also confirm the activation of other candidate RTKs identified in the phospho-proteome profiling assay but as the results showed, Axl is the RTK that is likely to have the greatest impact on c-Abl mediated functions. For downstream effectors, we identified Akt as target of the c-Abl-Axl axis. Akt has been shown to affect growth, survival and invasion downstream of Axl. It is still possible however to find other downstream effectors that are specific in the context of HCC in the future. Needless to say, based on the results obtained thus far, future studies should focus on establishing if there is a direct protein-protein interaction between c-Abl and Axl or if the interaction is indirect. The nature of interaction is important for a complete understanding of c-Abl function in HCC.

CHAPTER 3

INHIBITING C-ABL ACTIVATION ATTENUATES TUMOR CELL GROWTH AND PROLIFERATION *IN VIVO*.

3.1 Introduction

As pointed earlier, systemic therapy in HCC is very limited and extensive studies are being done either to comprehensively understand existing known target or to identify novel drug targets. The hope is that identification and characterization of key viable druggable targets will improve systemic therapy options for HCC in the clinic. Once such targets are identified it is crucial to test them out in relevant models that capture the phenotypes observed in the clinic for translational and drug development purposes.

Our in vivo studies here used a hydrodynamic delivery oncogene-induced mouse model. In the model, Figure 11, a Sleeping-Beauty transposon system with activated oncogene DNA (c-Met/β-Catenin) is hydrodynamically injected via tail vein. Transgenically, c-Met or β-Catenin alone cannot efficiently form tumors in mice but their co-transfection via tail vein injection will result in stable expression in liver hepatocytes and tumor formation after about 5 weeks. As mentioned earlier, β-Catenin (26%) and c-Met (40%) are two oncogenes that are found in HCC developmental pathways. In fact, at least 20% of HCC cases in the clinic arise from cooperation between activated β-catenin and c-Met as drivers of hepatocarcinogenesis. It has been proposed that accumulation of β-catenin is an alternate route to carcinogenesis as β-catenin positive HCC tend to have a distinct and unique phenotype. Since at least 20% of the HCC in the clinic arise from β-catenin and c-Met, our HCC mouse model recapitulates a disease phenotype that actually occurs in the clinic. Here we provide evidence that inhibition of c-Abl using Nilotinib, a second generation inhibitor slows HCC tumor growth, development and tumor cell proliferation.

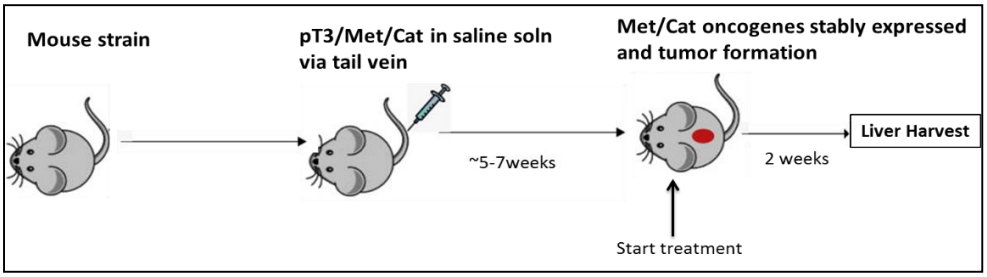

Figure 12. HCC liver cancer mouse model procedural schematic. Activated form of β-catenin (ΔN90-β-catenin) and c-Met (c-MET/CAT) are hydrodynamically injected via the tail. (Modified from Chen X et al) [53]

3.2 Materials and Methods

MET/CAT HCC model

Plasmids, encoding the Sleeping Beauty transposase, transposons, gaussia luciferase and human oncogenes c-MET (22.5μg) and CAT (22.5 μg activated ΔN90-βcatenin) were transfected into livers of age- and gender matched mice via tail-vein hydrodynamic delivery. The plasmids were in 10% v/w 0.9% saline solution. After 5 weeks post-transfection, mice were orally administered Nilotinib at 20 mg/kg in PEG 400 vehicle for 2 weeks. Following these 2 weeks, all mice were sacrificed and liver weight and body weight of each mouse were measured before livers were collected and stored for further analysis.[53]

Cell Lysis and Western blotting

Tissue lysates were prepared by lysing a pea-sized liver tissue in RIPA buffer supplemented with phosphatase and protease inhibitors. Protein fractions were then run on SDS-PAGE gels using protocols from antibody manufacturers.

Reagents

Primary antibodies directed against GAPDH, β-actin, pErk, pAkt (S473), pSrc, pCRKL were purchased from Cell signaling Technology (Danvers, MA). Antibodies directed against Axl and pAxl (Y772) were purchased from R&D systems. c-Abl antibody was purchased from Santa Cruz , CA and pc-Abl (Y412) was

obtained from Abcam. BrdU antibody was purchased from Invitrogen. Ki67 purchased from Fischer Scientific. AFP was purchased from Dako. Sorafenib was purchased MedKoo Biosciences. Nilotinib was purchased from LCLlabs.

3.3 Results

3.3.1 c-Abl activity is necessary for cell growth and proliferation *in vivo*.

To build more evidence that underscores the critical role of c-Abl in HCC in the clinic, we wanted to show that inhibition of c-Abl activity pharmacologically in vivo will slow down tumor growth significantly in a clinically relevant HCC mouse model. We had evidence to believe that c-Abl activity plays an important role in HCC progression based on cell viability and cell growth assays done in vitro earlier using Nilotinib. Specifically, cell growth and viability in vitro were determined by treating huh7 cells with Nilotinib (0-15 μM). The concentration ranges is the common range for cell viability studies with Nilotinib in the field. These assays indicated both qualitatively and quantitatively that Nilotinib slows down HCC growth and viability.

In vitro, all concentrations used were successful in slowing down growth of Huh7 cells. This inhibition was even more pronounced at 48 hours (Figure 13). Western blot was used to confirm that indeed Nilotinib was inhibiting growth by decreasing c-Abl kinase activity (Figure 13A). I*n vivo*, our results show smaller liver per body weight ratio in the Nilotinib therapy group than the vehicle therapy group with a statistical significance ($p<0.05$). Tumor foci were smaller and fewer tumors in the treated mice relative to the vehicle therapy group as determined by H&E staining (Figure13C). Signal for a cell proliferation marker ki67 showed less proliferation in Nilotinib-treated livers. AFP also confirmed the presence of more HCC tumors in vehicle versus Nilotinib-treated livers. Taken together, these results indicate that Nilotinib attenuates cell proliferation, tumor growth and tumor development in HCC.

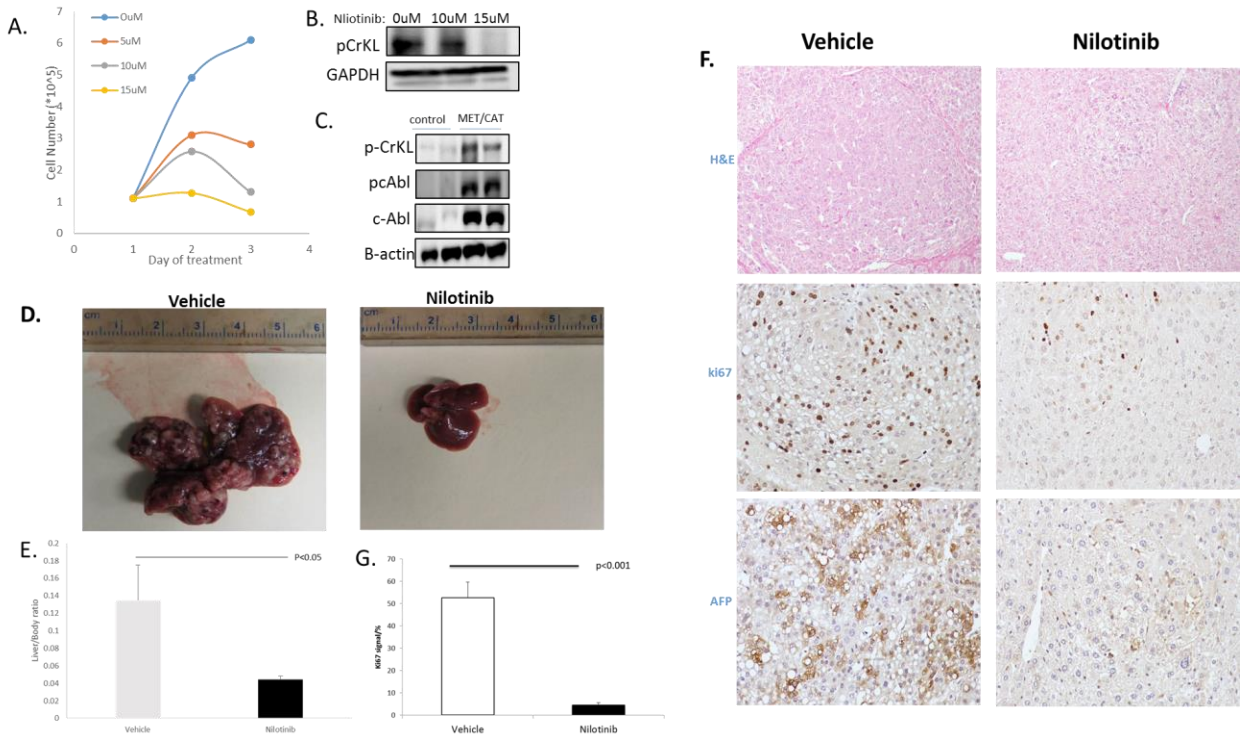

Figure 13. Activated c-Abl promotes tumor growth in HCC *in vivo*. A,B. Cell growth inhibition *in vitro* and decrease in c-Abl activity upon Nilotinib treatment. C. Met/Cat-induced HCC increase c-Abl levels. D,E. Gross images of vehicle and Nilotinib treatment mice livers and respective average liver/body ratios ($p<0.05$). F. Immunohistochemical analyses showing tumor formation, proliferation,HCC development as determined by H&E, ki67 and AFP stainings, respectively. G. ki67 % signal with Nilotinib treatment.

3.4 Discussion and Conclusions

For most kinases, it is the kinase activity on their targets that drives oncogenic function. Most kinases are therefore targeted pharmacologically by inhibitors that compromise or abrogate their catalytic activity. After showing evidence that c-Abl is important for HCC growth through gene silencing, we explored how inhibiting it at catalytic activation level will affect growth. There are many c-Abl inhibitors available in the clinic for leukemia but Nilotinib is the inhibitor that has been shown to be more specific for c-Abl.

Here, we reported that Nilotinib slows down HCC growth both *in vitro* and *in vivo*. In our cell-based models, Nilotinib reduces viability and cell proliferation. In our HCC mouse model, we see tumors as

early as 5 weeks after transfection of oncogenes but even after 7 weeks post-transfection, we did not see tumors with a naked eye in Nilotinib treated mice. Analysis, at a molecular level, reveals that proliferative capacity of tumors were tremendously diminished. AFP, which is a late-stage marker of HCC in the clinic, showed that Nilotonib treated mice have less advanced HCC tumors compared to vehicle. Even though, AFP specificity is usually between 50 to 85% in HCC patients, it is still tempting to say that this result indicates that Nilotinib slows down HCC development. What we can say for certain from this however is that Nilotinib robustly decreases tumor size, growth. As already mentioned, this model is relevant for significant percentage of HCC patients and these results give an indication that Nilotinib may be a relevant inhibitor for certain HCC patients especially if it used in targeted clinical trials. An interesting prospect in the future is to quantitatively investigate whether Nilotonib also causes tumor regression.

Considering the dramatic differences in tumor sizes and extent of proliferation between vehicle and Nilotinib therapy groups, these results are an encouraging sign that targeted therapy against c-Abl might be effective against certain HCC patients. Our confidence is boosted further by relevance of our HCC mouse model. B-catenin and c-Met are known oncogenes that on their own or in cooperation are found in human HCC. They have been shown to be associated with poor prognosis as well. However, there are more studies that need to be done to substantiate these results. One such study to confirm the role of *c-A*bl *in vivo* in the same HCC mouse model is to use a c-Abl liver conditional knockout and study how ablation of c-Abl affects tumor growth. If similar results are obtained, this will validate the importance of targeting Abl to inhibit HCC development.

CHAPTER 4

C-ABL KNOCKDOWN HAS SYNERGISTIC EFFECTS WITH SORAFENIB IN HCC INHIBITION *IN VITRO* AND *IN VIVO*

4.1 Introduction

In the 2007 Sorafenib HCC Assessment Randomized Protocol (SHARP) clinical trial, Sorafenib was found to inhibit liver tumor growth and their metastases, increasing the median overall survival by about 3 months compared to placebo. This result led to Sorafenib approval by the FDA. Even though 3 months does not appear to be a long time, for patients with intermediate and advanced HCC, this is their only chance to prolong their lives. Sorafenib increases survival by only 90 days and intrinsic resistance of HCC to chemotherapy may a contributing factor. Adverse effects of Sorafenib on the other hand have been touted to contribute significantly to its limited efficacy. Equally important is the acquired resistance to Sorafenib that arises from chronic treatment. Therefore current research in HCC drug development is focused on finding more effective drug targets or targets that have synergistic or additive effects with Sorafenib. Some of these combinations studies are being tested in current clinical trials.[51] Most clinical trials are targeted against kinases, some are antibody-based therapies and both are meant to additively compliment the anti-growth and anti-angiogenesis efforts by Sorafenib. To circumvent acquired resistance, some combinations are aimed at chemosensitizing Sorafenib-treated HCC tumor cells with upregulated compensatory pro-survival and growth pathways. In this light, it is also important to investigate and uncover the mechanism of synergism.

Here, we show that c-Abl inhibition synergistically works with Sorafenib in shrinking HCC tumor growth. We also identified key candidates whose activation changes significantly during the combination

treatment and this give us some insights into the possible mechanism of synergism between Sorafenib and c-Abl inhibition.

4.2 Materials and Methods

Hoescht Stain Apoptosis Assay

Scrambled and c-Abl knockdown huh7 cells were treated at similar confluency with sorafenib and vehicle for 48hrs. Cells were lifted, spun down and resuspended and fixed with Magic Solution containing the Hoescht stain. Solution were loaded into a multi-well micro slides and visualized under the microscope.

Cell Growth Assay

Equal number of cells infected with scrambled and target genes shRNA were counted and seeded in 12 well plates. Pictures were taken under the microscope as soon as soon attached (~4hours) and this was deemed time 0hrs. One set of scrambled and knock down cells were treated with Sorafenib and a corresponding set was treated with vehicle. More pictures were then taken after every 24 hours and after images were collected, the cells from the respective wells were then put in trypan blue and quantified using an automated cell counter.

4.3 Results

4.3.1 c-Abl knockdown enhances Sorafenib inhibition on growth and induction of apoptosis *in vitro*.

With evidence of a significant effect on the phenotype of HCC both *in vitro* and *in vivo*, it was interesting to explore the possibility of additive or synergistic effects between c-Abl inhibition and Sorafenib. c-Abl has never been shown to be a target of Sorafenib to our knowledge. An interesting observation was however made in Sorafenib treated cells; c-Abl expression increased steadily over time. An inference that could be made from this is that one of the countermeasures that HCC cells take when treated with

Sorafenib, is to upregulate other proteins, including c-Abl (Figure 16). Inhibiting c-Abl therefore appears like a rational strategy to enhance blockade of HCC growth by Sorafenib. What was not clear from this observation was whether this was a real change in c-Abl expression or just a spurious finding that needed to be confirmed.

Here, we wanted to investigate the effect of combining c-Abl inhibition with Sorafenib treatment in HCC both at cellular and molecular levels. It is important to note that these experiments were initially done using shRNA against c-Abl since Nilotinib also inhibits activity of c-Abl's close relative, Arg. Any results obtained from this study therefore can be pinned down to exclusively to c-Abl function, not Arg or both Arg and c-Abl.

Cell growth was assayed with combination treatment of c-Abl inhibition and Sorafenib and cell numbers were quantified. Cell growth was more potently inhibited with combination treatment compared to either c-Abl knockdown or Sorafenib treatment alone. Even though growth continued steadily for single treatments, albeit still less than vehicle treatment, growth actually decreased after 48 hrs for combination treatment. It was also observed that there are more floating cells in the combined treatment group relative to single treatments (Figure 14). A follow up on this observation was an apoptosis assay using a Hoescht stain assay. The experiment was set up in the same manner as the growth assay and after 48 hours, cells were stained with the Hoescht dye. We found that there is more cellular fragmentation, which is related to DNA fragmentation, with combination treatment using two different shRNas compared to Sorafenib treatment alone. Since cellular fragmentation is a hallmark of apoptosis, these results suggest that there is more apoptosis when Sorafenib is combined with c-Abl inhibition. The observed increase in apoptosis was also significant ($p<0.05$) for Sorafenib and Abl knockdown combination treatment but not scrambled shRNA and Sorafenib (Figure 14).

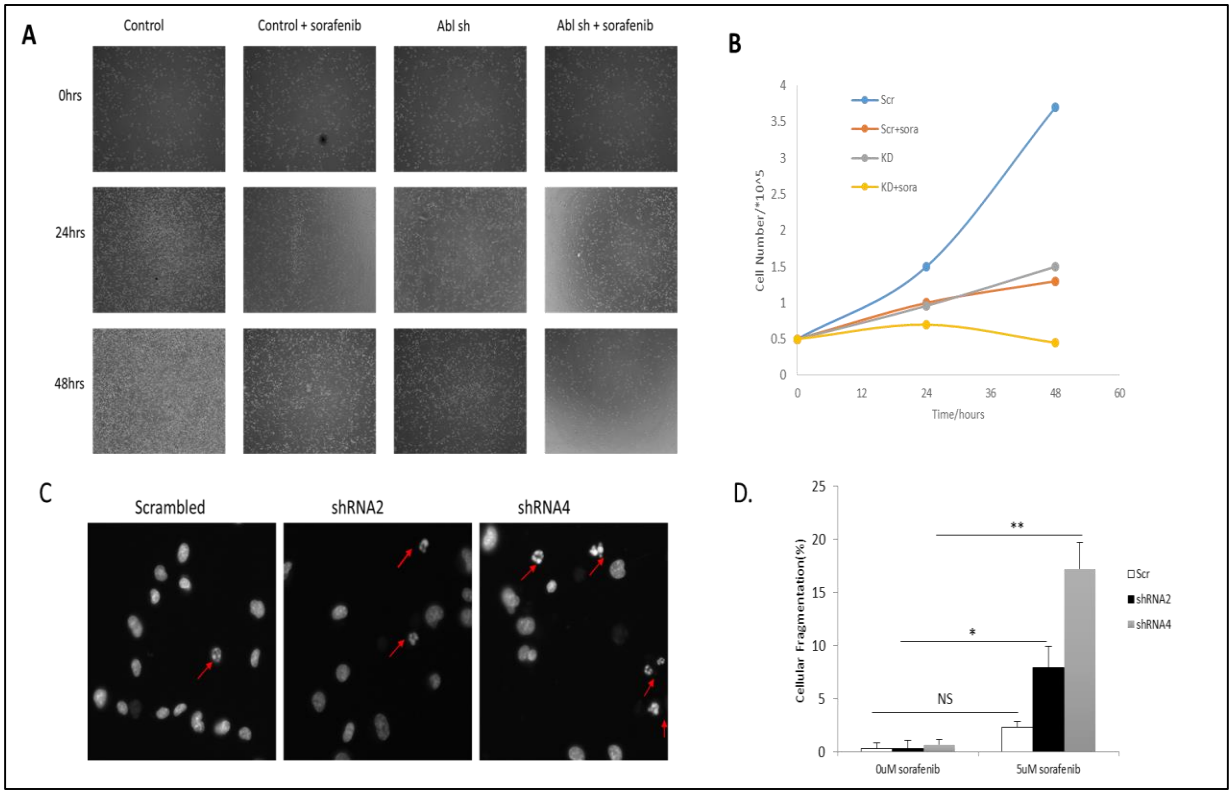

Figure 14. c-Abl inhibition sensitizes HCC cells to sorafenib treatment and sorafenib-induced apoptosis in vitro. A, B. Qualitative and quantitative analyses of cell growth in control and c-Abl knockdown cells with and without sorafenib combination treatment as seen under the microscope at various time points. C. Hoescht assay visual analysis of apoptotic events exhibited by cellular fragmentation of control and c-Abl knockdown cells with and without sorafenib combination treatment. D. Quantitation of extent of cellular fragmentation between single and combination treatments with statistical analysis. *NS=not significant, *=p<0.05, **=p<0.01*

4.3.2 c-Abl knockdown enhances Sorafenib-mediated HCC cell growth inhibition *in vivo*.

To see if there is cooperation between c-Abl inhibition and Sorafenib in HCC holds *in vivo*, we conducted an experiment in our xenograft mouse model. $5*10\text{^}6$ scrambled shRNA and c-Abl huh7 cells were injected to the left and right flanks of SCID-bg mice, respectively, and allowed to grow for 6 days. The mice were divided into 2 sets of groups; one set comprised mice injected with scrambled shRNA treated with and without Sorafenib via oral gavage. Another set, consisted of mice injected with knock down cells treated with and without Sorafenib. The treatment was carried out for seven days before the mice were sacrificed and the xenografted tumor cells harvested for further analysis.

Our *in vivo* results show consistently enhanced inhibition of cell growth in the combination therapy group compared to the vehicle group. Combination treatment resulted in tumor volume becoming virtually static during the 7-day period of treatment. Sorafenib treatment of scrambled shRNA and c-Abl knockdown alone did cause a decrease in tumor volume compared to scrambled shRNA treated with only vehicle but tumor sizes continued to increase during the 7-day period. Tumor weights also showed a similar trend, with the combination therapy group with smallest average tumor mass. Comparisons of both tumor volumes and weights between groups were statistically significant. Specifically, there was statistical significance between Abl knockdown and combination treated cells ($p<0.01$) and an even higher significance between Sorafenib treated scrambled cells and combination treated cells ($p<0.005$). Only the comparison between vehicle and Sorafenib treatment did not reach significance (Figure 14).

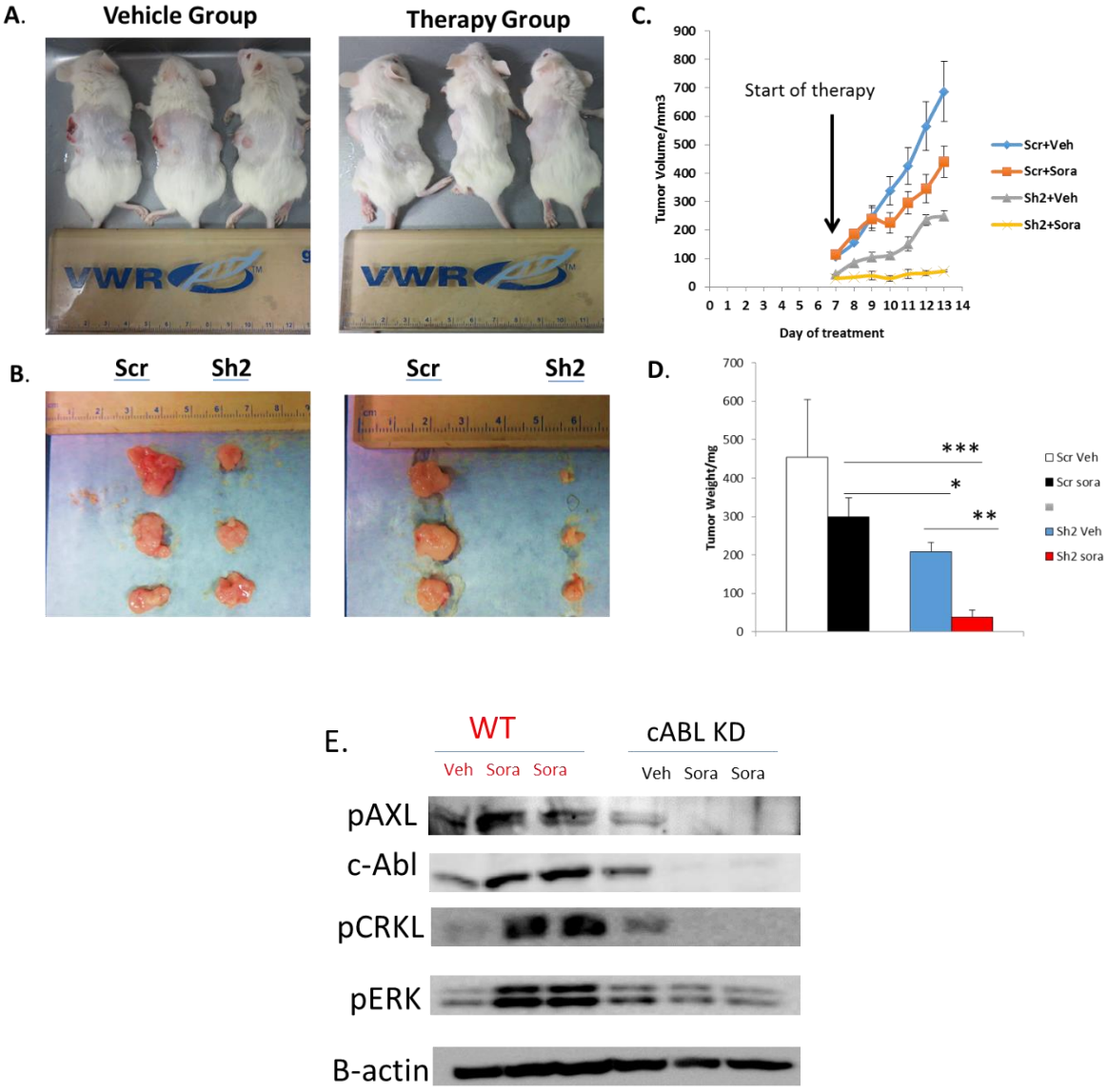

Figure 15. Figure 10. c-Abl inhibition sensitizes HCC cells to sorafenib treatment in vivo. A. SCID-bg mice injected on the flanks with control (left) and knockdown cells (right) with and without sorafenib. B. Gross images of tumors after harvest control (left) and knockdown cells (right) with and without sorafenib. C, D. Tumor volumes and weights averages of control (left) and knockdown cells (right). E. Immunoblot showing levels of c-Abl, pCrkL, pAxl, pErk in xenograft mice in the combination treatment experiment. **=p<0.05, **=p<0.01, ***=p<0.005*

4.3.3 Sensitization of c-Abl KD cells to Sorafenib treatment occur through a c-Abl-Axl axis

Sorafenib is a multi-kinase inhibitor that targets pathways critical for HCC development. Nevertheless, cells tend to mount defensive mechanisms to circumvent the Sorafenib assault. Even though some of the pathways that switch the addiction have been proposed, inhibiting those pathways has not always yielded the expected the chemosensitization especially in the clinic. It stands to reason that there are other pathways whose signatures and profiles are changed when HCC tumors are treated with Sorafenib.

One of the outstanding questions in combination experiments was whether Sorafenib affects c-Abl activity. c-Abl was shown not to be a target of Sorafenib before but since we didn't know this would be the case as well in our context, we wanted to check. To begin to answer this question, we used xenograft tissue to uncover the effect of Sorafenib on c-Abl and we did not expect c-Abl to be affected by Sorafenib. If Sorafenib affected c-Abl, we expected it to decrease its activity since Sorafenib inhibits a number of kinases. Our results however, show a surprising finding (Figure 15 E). Sorafenib treatment resulted in an increase not only in c-Abl levels but also c-Abl activity levels, as indicated by pCrkL, in scrambled control cells. Sorafenib treatment also led to an increase in pERK levels and this was consistent with other in vivo studies that showed activation of pERK after several days of treatment. Because we had found Axl levels to be affected by c-Abl knockdown *in vitro*, we also tested the levels of pAXL in this *in vivo* model. Our findings show that Axl activation increases when xenograft tumors were treated with Sorafenib. However, when c-Abl is knocked down, we did not see an increase pAxl or pCrkL. We also observed tenuous levels of pERK when c-Abl was knocked down upon Sorafenib treatment.

With these results in hand, we decided to do a follow up study in which we treated huh7 cells *in vitro* with Sorafenib for 5 days and measure effects on c-Abl and its targets. We found c-Abl levels to be elevated in a time dependent manner. The c-Abl activation levels didn't increase right away (Figure16).

eventually they started to increase at the 5th day of the experiment as well. The levels in c-Abl expression were also matched by a steady increase in pAkt and pErk. Since we found Axl phosphorylation to be higher when scrambled cells, we also tested pAXL levels *in vitro* after treatment with Sorafenib for several days. Again, we observed a consistent increase in pAXL levels with

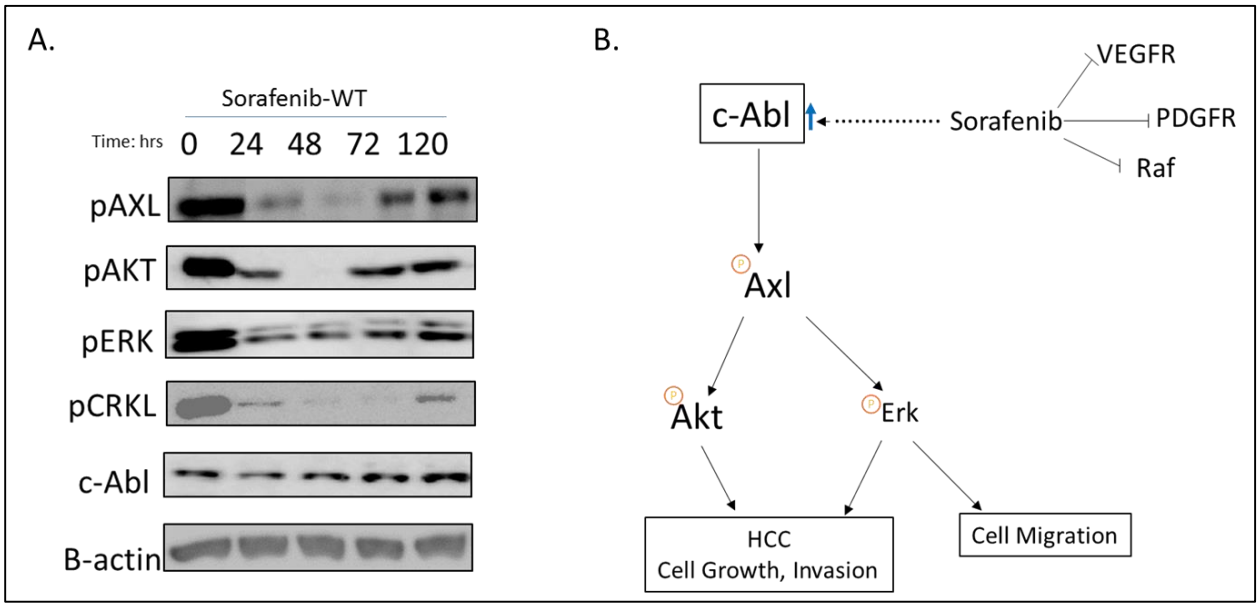

Figure 16. Sensitization of c-Abl KD cells to Sorafenib treatment occur through c-Abl-Axl axis. A. Immunoblot showing pAxl levels initially suppressed with sorafenib treatment which then start to increase with in a time dependent manner. This is matched by a concomitant rise in c-Abl levels and activity and other downstream targets of Axl. B. A shematic model showing how through Axl c-Abl elicits its function. The model also proposes how c-Abl inhibition can rescue sofarenib-induced resistance.

It was inhibited during the first 3 days of treatment. On the 5th day, c-Abl activity started to increase. The same trend was also observed for pAxl, pAkt, pErk levels, with 5th showing signs of recovery (Figure 16).

4.4 Discussion and Conclusions

Combination treatments have had huge success in treating many solid tumors as first line of treatment or as second line when tumors become refractory. In other cancers, combinations include regimens with both cytotoxic and targeted inhibitors. However, since cytotoxic drugs are virtually never used for HCC treatment for lack of therapeutic benefit, combination strategies that include targeted drug cocktails seem to be the only other option. A quick glimpse at ongoing trials in the clinicaltrials.gov database reveals such attempts are being made. For instance, inhibitors against IGF-1R, Mek1, mTOR are being tested in trials in combination with Sorafenib. Combination treatment of HCC with Sorafenib and c-Abl has never been explored to our knowledge. In this report, we investigated the synergy between Sorafenib and c-Abl inhibition in HCC treatment. Our findings, show that combination treatment is better at inhibiting growth. This observation was corroborated by an increase in apoptosis in the double treatment group compared to single treatment, either for c-Abl inhibition or sorafenib administration alone. More importantly, our results show that this enhancement of growth inhibition or cell death is also observed *in vivo*, which improves the utility of our combination strategy involving c-Abl. We also studied how some of these observations could be explained at a molecular level. Not only, have we shown that Sorafenib treatment increases c-Abl expression and activation in this study, we have also identified Axl as a target of c-Abl in HCC. With increase in c-Abl activation or expression, it is clear to see why combination treatment resulted in sensitization. It is possible that c-Abl expression is a compensatory mechanism for cells to regain growth or survival phenotype upon Sorafenib treatment and thus knocking c-Abl denies the HCC cells this avenue. What's more is that continued treatment with Sorafenib also increases activation of Axl and activation of its downstream targets Erk and Akt. This is

consistent with expectations because similar observations have been made when dual anti-growth and anti-angiogenic drugs are used to treat tumors. Akt and Erk phosphorylation have been widely proven to be the upregulated resistance downstream effectors after chronic treatment of HCC with sorafenib.[12, 13] Sutinib, a multi-kinase inhibitor that targets similar proteins as Sorafenib, with the exception of only Raf isoforms, has also been shown to increase Akt phosphorylation after chronic treatment in renal cell carninoma (RCC). More over, treatment of RCC with Sunitinib also results in upregulation of Axl expression and activation and this has been proposed as the mechanism by which RCC tumors acquire resistance to sunitinib. In HCC, our results show that Axl activation recovers from inhibition after cycle of repeated Sorafenib treatment *in vitro* and *in vivo*. This was matched by concomitant recovery in c-Abl activation as well. We cannot be absolutely certain at this point how the communication of c-Abl and Axl happen with and without the inhibitor or if the increase in c-Abl expression and activation directly influence the activation of Axl. But c-Abl does influence Axl in one way or another and this influence appears to mediate the oncogenic function of c-Abl in HCC. And since there is a lag in c-Abl and Axl activation response, we can predict that c-Abl is a potential critical player in Sorafenib-induced resistance and its inhibition removes cell's ability to compensate for pathways that are inhibited by Sorafenib. Based on these observations, we can put forward a model that involves c-Abl and Axl and the processes that we have shown these two kinases control. Our model involves Axl as a downstream target in HCC and activation of Axl by c-Abl leads to signaling of proliferative and migratory pathways using Erk and Akt as effectors (Fig 16 B). When Sorafenib is used to treat HCC, through a mechanism that we do not understand at the moment, there is increased expression and activation of c-Abl. This activation in turn is relayed down to Axl and downstream signaling pathways.

Even though we have good reasons to be believe that the observed phenotypes do involve signaling through an axis involving c-Abl and Axl, future studies need to be done to clearly establish the interaction and signaling crosstalk between c-Abl and Axl in HCC in promoting growth, pre-metastatic

processes and response to chemotherapy. Specifically, domain or site-directed mutation studies involving domains or specific amino acids of c-Abl and or Axl need to be conducted to determine if there is physical protein-protein interaction between Axl and c-Abl. These domains are likely to be in the Sh2 domain of c-Abl and certain tyrosines in Axl. Axl does not have documented proline rich motifs or SH3/SSH2 domains that may play a role in protein-protein interaction. c-Abl on the other hand does have an SH2 domain that recognizes phosphorylated tyrosines of other proteins.

There is also need to confirm the role of other RTKs that were identified during the RTK assay to build a complete picture of how c-Abl might elicit its oncogenic function in HCC development. Another important future study to conduct is a combination study involving Sorafenib and Nilotinib since it is Nilotinib is the actual inhibitor that will be used in the clinic should c-Abl be a viable clinical target. With those future studies we will able to build a complete picture that gives important insights for rational therapeutic strategies that will help with efforts to develop single agents for HCC or combining those agents with Sorafenib.

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VITA

Lennox Chitsike was born in Harare, Zimbabwe. He graduated from Hamilton College with a Bachelor of Arts honors in Biochemistry/Molecular Biology in 2013. For his senior thesis, Lennox worked in Dr. Myriam Cotten's lab where he studied the structure-function relationships of P1 on tumor cell models. After graduation he continued to work in her lab consolidating the results of his thesis.

In the fall of 2014, Lennox entered the Molecular Biology Program at Loyola University Chicago and he joined the laboratory of Dr. Wei Qiu where he studied the role of c-Abl in Hepatocellular Carcinoma (HCC) development.