

ABL1 is overexpressed and activated in hepatocellular carcinoma

Lennox Chitsike¹, Xianzhong Ding², Peter Breslin³ and Wei Qiu^{1*}

Departments of ¹Surgery andOncology Institute,²Pathology, ³Molecular/Cellular Physiology,
Loyola University Chicago Stritch School of Medicine, 2160 South 1st Avenue., Maywood, IL
60153, USA.

Emails of authors:

LC: lennoxpc@gmail.com;XZD: xianzhong.ding@lumc.edu;PB: pbresli@luc.edu; WQ:
wqiu@luc.edu.

Disclosures: The authors have no conflicts to disclose. All authors agreed on the submission.

Author contributions:

LC performed experiments, analyzed data and wrote the paper.

XZD analyzed data.

PB wrote the paper.

WQ designed and supervisedexperiments, analyzed data and wrote the paper.

*Correspondence:

Wei Qiu, Ph.D., 2160 South 1st Avenue., Bldg. 112 Rm. 338, Maywood, IL

Email: wqiu@luc.edu;

Abbreviations: HCC, hepatocellular carcinoma; Immunohistochemistry, IHC; Tissue Microarray,
TMA; CAT, constitutively-active β -catenin.

Abstract

Background & Aims: ABL1 is a non-receptor tyrosine kinase of the Abelson (Abl) family, which plays an important role in cell growth, survival, invasiveness, adhesion, and migration. Despite the well-known role of BCR-ABL in hematologic malignancies, the role of ABL1 in hepatocarcinogenesis remains unknown. In this study, we want to determine if ABL1 is dysregulated in HCC.

Methods: We analyzed *ABL1* mRNA in two cohorts of HCC databases from *Oncomine*. In addition, we performed immunohistochemistry (IHC) to examine the expression of ABL1 and p-ABL1 (Y412) in HCC tissue microarray (TMA). Further, we examined ABL1 expression and activity in oncogenes cMET/ β -catenin-induced mouse HCC model.

Results: We found that *ABL1* mRNA is overexpressed in over 50% of HCC. We also found that ABL1 protein expression is upregulated or activated in about 35% HCC specimens compared to adjacent normal liver. In addition, we found that ABL1 protein expression is upregulated and activated in cMET/ β -catenin-induced HCC.

Conclusion: ABL1 is overexpressed and activated in human and mouse HCC. ABL1 might be a potential therapeutic target for HCC.

Key words: ABL1; Hepatocellular carcinoma; MET/CAT; CRKL

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most aggressive form of liver cancer, comprising 83% of all liver cancer cases. It is the 5th most common malignancy globally and is second in total cancer-related deaths annually (1). Even though it was formerly 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 USA in the past decades, with the rates more than doubling in the US (2). The overall survival of patients with HCC is less than 12%, and most patients with HCC have limited treatment options. Currently the most effective targeted therapeutic agent for advanced HCC, Sorafenib, only increases survival in patients with advanced HCC from 7.9 months to 10.7 months (3). Thus there is an urgent need to develop new and more effective therapeutic strategies and agents to treat HCC.

HCC is a very complex disease the etiology of which originates 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(4). HCC progresses **is** due to changes in a number of genetic and epigenetic profiles that affect key pro-oncogenic pathways. Many molecular analyses have shown that certain risk factors increase genomic instability through telomere shortening and chromosomal defects(5). β -catenin is one of the key mediators of Wnt signaling which, together with MET, ErbB, PI3K, VEGFR, Hedgehog, and FAK, represent key proteins that have been implicated in HCC(6-8). Even though these etiological factors have been identified and validated, gaps still exist in our understanding of how they can be fully exploited therapeutically 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 are therefore paramount for the development of more efficacious drugs.

ABL1 is a non-receptor tyrosine kinase of the Abelson-like (Abl) family. It is mostly known for its involvement in the Philadelphia chromosome, which result from the translocation of the short arms of chromosomes 9 and 22, creating a fusion 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 (9, 10). Even though c-Abl has been notoriously known for a long time now for its role in leukemias harboring the Philadelphia chromosomes, relatively recent evidence has shown that ABL1 plays a role in the progression of solid tumors by an independent mechanism not involving any fusion oncoproteins (9). Such a mechanism has mainly been attributed to copy number amplifications, mutations or catalytic activation resulting from hyperactive receptor tyrosine kinases or growth factor signaling. A number of immunohistochemical studies have demonstrated that c-Abl expression is upregulated in a number of cancers including brain, lung, ovarian, colon cancers, and others (9). Indeed, a number of studies show that ABL1 plays an oncogenic function in many solid tumors (9, 11-15). However, the role of ABL1 in hepatocarcinogenesis has not been established. Recently ABL2, another member of ABL kinases, was reported to be overexpressed in about 37% of HCC cases, and this was associated with poor prognosis for these patients (16). However, it is not known whether ABL1 is dysregulated in HCC.

In this study, we show that *ABL1* mRNA is overexpressed in two cohorts of HCC in *Oncomine* databases. In addition, we performed immunohistochemistry (IHC) and found that ABL1 protein expression is upregulated or activated in about 35% of HCC specimens compared to normal liver. Further, we found that ABL1 protein expression is upregulated and activated in cMET/ β -catenin-induced HCC. Altogether, our data indicate that ABL1 is overexpressed and activated in human and mouse HCC. Thus ABL1 might be considered a potential therapeutic target for HCC therapy.

2. MATERIALS AND METHODS

2.1 *ABL1* mRNA expression analysis

The *Oncomine*TM platform (Thermo Fisher, Ann Arbor, MI) was used for analysis and visualization. mRNA expression of *ABL1* in Roessler liver and Wurmbach liver cohorts were analyzed.

2.2 Immunohistochemical (IHC) Staining

Human colon tissue micro array (TMA) HLiv-HCC150CS was purchased from US Biomax (Rockville, MD, USA). HLiv-HCC150CS contains 75 cases of HCC and 75 cases of normal adjacent tissues. IHC was performed as previously described (7, 17, 18). Cells with positive staining were scored in at least 5 fields at 400× magnification and reported as mean $\pm$ SD.

The anti-ABL1 antibody (#SC-131) was purchased from Santa Cruz (Dallas, Texas) and the anti-p-c-ABL antibody (#PA5-39687) was purchased from Invitrogen (Carlsbad, CA).

2.3 IHC scoring criteria

The IHC signals were quantified visually. For ABL1, both cytoplasmic and nuclear localization were considered positive. The staining is scored as – (0, no signal), + (1, weak signal), ++ (2, moderate signal), +++ (3, strong staining) by two independent observers including a pathologist from Loyola University Chicago, masked to patient outcome; a sample was rated as positive if it showed at least 1% of cells with a staining score ≥ 1 (19).

2.4 Western Blotting

Western blotting was performed as previously described (7, 17, 18). Primary antibody against ABL1 (#2862), p-ABL1 (Y412) (#2865), p-CRKL (#3181) and CRKL (3182) were purchased from Cell Signaling (Danvers, MA). The β -actin antibody (#A5441) was purchased from Sigma-Aldrich (St. Louis, MO).

2.5 MET/CAT HCC Model

All animals received humane care according to the “Guide for the Care and Use of Laboratory Animals”. The procedures for all animal experiments were approved by the Institutional Animal Care and Use Committee at Loyola University Chicago. The mice were housed in micro-isolator cages in a room illuminated from 7:00 AM to 7:00 PM (12:12-hr light-dark cycle), and allowed access to water and chow *ad libitum*. For the MET/CAT-induced HCC model (7, 17, 18), 55µg of total plasmids, encoding the Sleeping Beauty transposase (HSB2) and transposons with oncogenes MET/CAT and gaussia luciferase (Gluc) (22.5µg pT3-EF1α-c-MET(human) + 22.5µg pT3-EF1α-ΔN90-β-catenin (human)+ 5µg pT3-Gluc+ 5µg HSB2), were injected hydrodynamically into age- and gender-matched mice. Mice were maintained on the standard diet and sacrificed after 7 weeks. The mouse livers were collected for western blotting.

2.6 Statistical Analysis

Statistical analysis was performed using *GraphPad Prism V* software. Data are presented as mean $\pm$ standard deviation (SD). Statistical significance was calculated using the Student's t-test. $P < 0.05$ was considered to be significant. The means $\pm$ SDs are shown in the figures where applicable.

3. RESULTS

3.1 *ABL1* mRNA is overexpressed in human HCC patients.

We first analyzed mRNA expression of *ABL1* using the *Oncomine* platform. We found increased expression of *ABL1* mRNA in human HCC specimens compared to normal livers in two HCC cohorts, Wurmbach Liver and Roessler Liver. In both databases, *ABL1* mRNA expression was increased by about 1.49-fold in human HCC specimens compared to normal livers (Fig. 1 A&B). Importantly, *ABL1* mRNA was increased in 68% (15/22) of HCC samples in Roessler Liver and

57% (20/35) of HCC samples in Wurmbach Liver. Notably, the over-expression gene rank of *ABL1* is 65 (in the top 1%) and 1719 (in the top 9%) in Roessler Liver and Wurmbach Liver databases, respectively. In general, these data indicate that *ABL1* mRNA is overexpressed in human HCC.

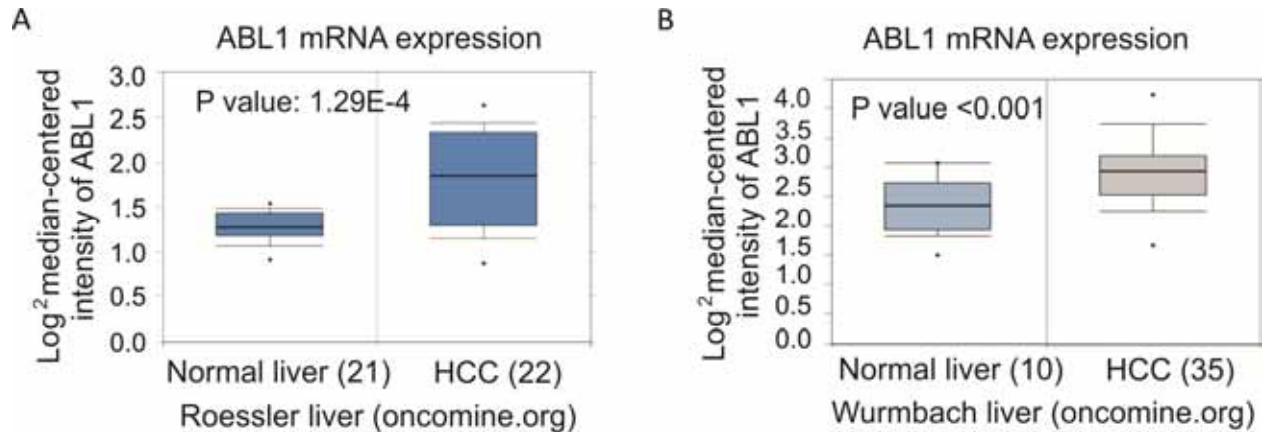


Figure 1. *ABL1* mRNA is overexpressed in human HCC patients.(A) *ABL1* mRNA expression in human normal liver and HCC from Roessler liver database (oncomine.org). (B) *ABL1* mRNA expression in human normal liver and HCC from Wurmbach liver database (oncomine.org).

3.2 *ABL1* protein is overexpressed in human HCC patients.

ABL1 can be regulated through different regulatory mechanisms at the transcriptional, translational, and post-translational levels. It remains unknown whether *ABL1* protein is dysregulated in human HCC samples. To address this question, we performed IHC of *ABL1* using HCC tissue microarray (TMA) purchased from US Biomax. We found that *ABL1* protein is mostly (90%, 67/75) absent or expressed at a low level in normal liver tissues (Fig. 2, A&B). In the contrast, *ABL1* is highly expressed in 30% (23/75) of HCC specimens (Fig. 2, A&B). Notably, *ABL1* staining was observed in both cytoplasmically and in the nucleus in HCC cells (Fig. 2A), which is consistent with previous reports (9). These data indicate that *ABL1* protein is overexpressed in a subset of human HCC.

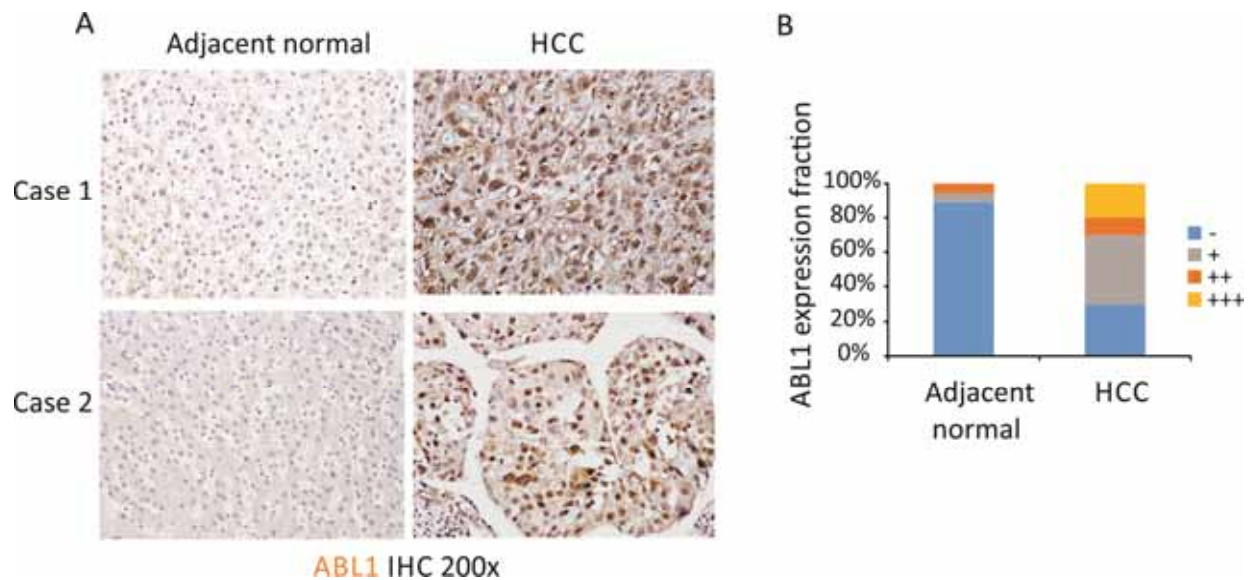


Figure 2. ABL1 protein is overexpressed in human HCC patients.(A) Representative photographs of IHCstaining of ABL1 in human adjacent normal liver and HCC. (B) Quantification of expression of ABL1 in a human HCC TMA.

3.3 p-ABL1 (Y412) expression is increased in human HCC patients.

ABL1 kinase activity is increased by diverse physiological stimuli including integrin activation and growth factor stimulation(e.g. PDGF)(20). Phosphorylation at Tyr412, which is located in the kinase activation loop of ABL1, is required for its kinase activity(21). To demine if ABL1 is activated in human HCC samples, we also performed IHC for p-ABL1(Y412) using the same HCC TMA from US Biomax. We found that p-ABL1 protein is also largely absent or expressed at a low level in normal liver tissues (93%, 70/75)(Fig. 3A). On the other hand, p-ABL1 (Y412) is highly expressed in HCC specimens (35%, 26/75). Notably, mostof the p-ABL1 staining was observed in the HCC samples showing high expression of total ABL1 (86%, 22/26).

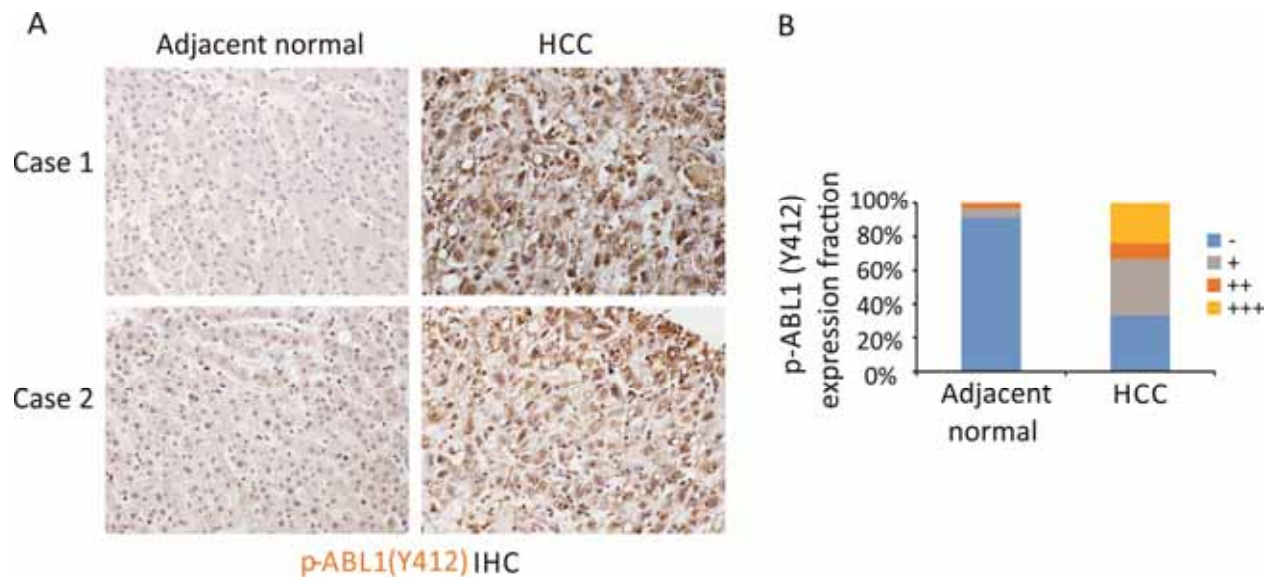


Figure 3. p-ABL1 expression is increased in human HCC patients.(A) Representative photographs of IHCstaining of p-ABL1 (Y412) in human adjacent normal liver tissue and HCC. **(B)** Quantification of expression of p-ABL1 (Y412) in a human HCC TMA.

3.4 ABL1 protein is overexpressed and activated in mouse HCC.

We further examined expression and activation of ABL1 in a mouse HCC model. The oncoproteins c-MET and β -catenin play critical roles in hepatocarcinogenesis. Co-activation of c-MET and β -catenin often occurs in HCC (22). It has been reported that 60% of HCC specimens containing activated c-MET also show activation of β -catenin, and over 60% of HCC samples carrying mutant β -catenin contain activated c-MET (22). In addition, co-delivery of both c-MET (MET) and constitutively-active β -catenin ($\Delta N90$ - β -catenin, exon 3 deleted, CAT), but not MET or CAT alone, into mouse livers using the Sleeping Beauty Transposon system efficiently induces HCC within several weeks (22-24). Therefore, this model (referred to here as MET/CAT) is useful to study the functions of genes in hepatocarcinogenesis because of its clinical relevance and efficiency in HCC induction. To determine whether ABL1 is also increased and activated in mouse HCC models, we examined protein expression of phosphorylation of CRKL (a direct target of ABL1 (25)), CRKL, p-ABL1 (Y412) and ABL1 in control normal liver and

MET/CAT-induced liver tumors. We found that expression of p-CRKL, p-ABL1 and ABL1 was all increased in MET/CAT-induced tumors (Fig. 4), which suggest ABL1 is overexpressed and activated in MET/CAT-induced HCC.

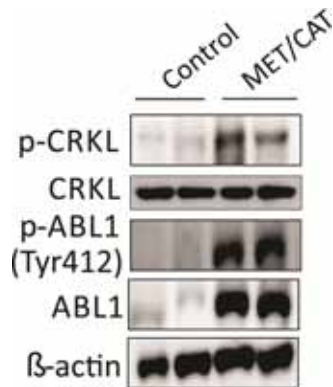


Figure 4. ABL1 is overexpressed and activated

in MET/CAT-induced mouse HCC.Protein

expressions of p-CRKL, CRKL, p-ABL1, ABL1 and β -actin in the livers of the mice treated with control and MET/CAT for 7 weeks.

4. DISCUSSION

The role of ABL1 in HCC remains unknown. In this study, we analyzed two separate databasecohorts of HCC. We found that that *ABL1* mRNA is overexpressed in HCC. In addition, we found that ABL1 protein is upregulated or activated in about 35% of HCC specimens by immunohistochemistry.We also found that ABL1 is overexpressed and activated in MET/CAT-induced mouse HCCs. Altogether, the data presented here indicate that ABL1 is overexpressed and activated in a subset of HCC. Therefore, ABL1 might be a potential therapeutic target in HCCpatients who have activated or high expression of ABL1.

It is notable that *ABL1* mRNA expression was increased by over 3-fold in about 30% of HCC samples despite an overall 1.5-fold increase among all HCC samples compared to normal livers. Consistent with this finding, an intense staining signal for both ABL1 and p-ABL1 was observed in 30% of HCC samples.This suggests that ABL1 is highly overexpressed in some HCC samples. It is not known whether*ABL1* mRNA level is correlated with protein level in HCC samples, as we are not able to examine ABL1 protein expression level from the *Oncomine* database nor measure *ABL1* mRNA levels from TMA tissues. However, most HCC sampleswith

high p-ABL1 expression showed high expression of ABL1, suggesting that the activation of *ABL1* might be the result of transcriptional or translational regulation in HCC samples. Five percent of HCC samples showed high p-ABL1 but low ABL1 staining, suggesting that ABL1 can be activated by ligands without an increase in total protein.

ABL1 can be activated by multiple mechanisms, including copy number amplifications, transcriptional regulation, mutations and catalytic activation resulting from hyperactive receptor tyrosine kinases or growth factor signaling(26). Copy number amplifications can lead to an increase in the expression of ABL1 at mRNA level, which in turn enhances the activation of Abl kinases. However, as *ABL1* copy number amplification is rare (7/366, 1.9% in TCGA database) in HCC, it does not seem to be a major mechanism to contribute to ABL1 activation. In addition, *ABL1* mutation only happened in 1/366 TCGA HCC specimens, suggesting mutations also does not contribute to ABL1 activation in human HCC. On the other hand, *ABL1* mRNA expression was increased by over 3-fold in about 30% of HCC samples. It is possible that *ABL1* is regulated at transcriptional level. However, little is known about the promoter elements and transcription factors that regulate the expression of *ABL1*. On contrast, It is reported that *ABL1* mRNA translation is silenced by microRNA-203 (miR203)(27), and sequence analysis suggests additional posttranscriptional regulators for ABL1 (miR196) and ABL2 (miR26 and miR1297)(10). Thus, increased *ABL1* mRNA in HCC may be due to posttranscriptional regulation by these miRNAs. ABL1 can be activated by integrins, hyperactive adhesive receptors or mitogenic growth factors, such as EGFR, PDGFR, IGF-1R and c-Met(10). It has been shown that HGF/MET can directly bind to ABL and activate ABL in mouse mammary tumors and breast cancer cells (28). Indeed we found that ABL1 was activated in MET/CAT-induced tumors (Fig. 4), suggesting MET might directly activate ABL1 in mouse hepatocytes.

5. CONCLUSION

In this study, our data indicate that ABL1 is overexpressed and activated in human and mouse HCC. Thus ABL1 might be considered a potential therapeutic target for HCC therapy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

ACKNOWLEDGEMENTS

We thank Drs. Jiwang Zhang, Mitchell F. Denning, Nancy Zeleznik-Le and Manuel Diaz (all from Loyola University Chicago) for their helpful discussions and advice. This work is supported in part by AASLD Liver Scholar Award (W. Qiu), NIH R03CA195183(W. Qiu), R03CA184652 (W. Qiu) and R01CA197128 (W. Qiu).

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