UNDER PEER REVIEW

Original Research Article

Short title: ABL1 is overexpressed in HCC

UNDER PEER REVIEW

ABL1 is overexpressed and activated in hepatocellular carcinoma

UNDER PEER REVIEW

Abstract

Background & Aims: There is an urgent need to develop new and more effective therapeutic strategies and agents to treat hepatocellular carcinoma (HCC). 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; Activated; Hepatocellular carcinoma; MET/CAT; CRKL

UNDER PEER REVIEW

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most aggressive form of liver cancer, comprising 83% of all liver cancer cases (1). 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 US 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 (2). 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 then progresses 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 singling 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.

UNDER PEER REVIEW

ABL1 is a non-receptor tyrosine kinase of the Abelson-like (Abl) family (9). It is mostly known for its involvement in the so-called 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 Philadephia chromsomes, 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. This information is necessary in order to establish whether ABL1 ought to be targeted to treat 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 adjacent normal liver. Further, we found that ABL1 protein expression is upregulated and activated in cMET/β-catenin-induced HCC. Altogether, the data presented indicate that ABL1 is overexpressed and activated in human and mouse HCC. Thus ABL1 might be considered a potential therapeutic target for HCC therapy.

UNDER PEER REVIEW

2. MATERIALS AND METHODS

2.1 *ABL1* mRNA expression analysis

The *Oncomine* ™ 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 ± 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).

UNDER PEER REVIEW

2.5 MET/CAT HCC Model

All animals received humane care according to the "Guide for the Care and Use of Laboratory Animals" (http://oacu.od.nih.gov/ac_cbt/guide3.htm). 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), 55 µg 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 ± standard deviation (SD). Statistical significance was calculated using the Student's t-test. $P < 0.05$ was considered to be significant. The means ± 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 (http://www.oncomine.com). 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

UNDER PEER REVIEW

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.

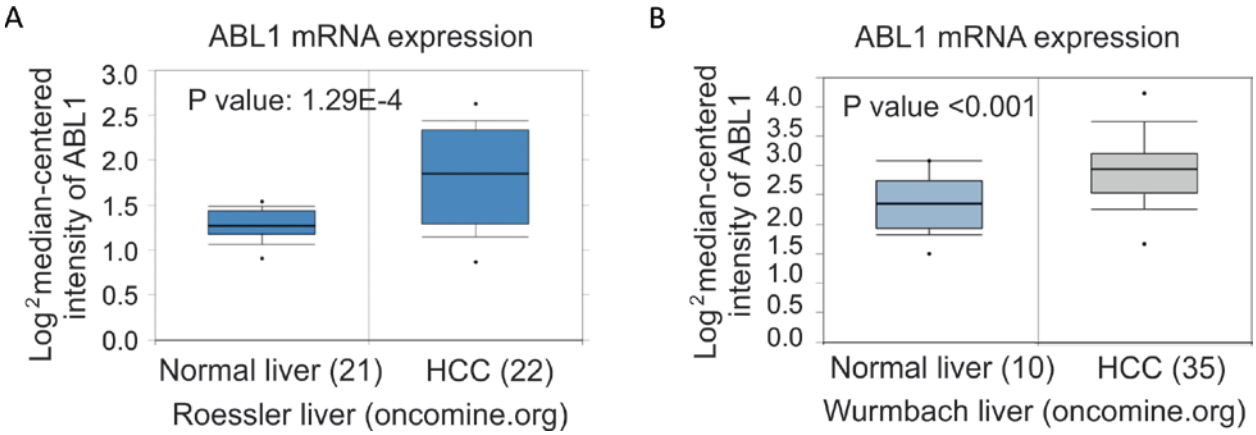

Figure 1. *ABL1* mRNA is overexpressed in human HCC patients. (A) *ABL1* mRNA expression in human normal liver and HCC from Roessler liver database (oncomine.org). **(B)** *ABL1* mRNA expression in human normal liver and HCC from Wurmbach liver database (oncomine.org).

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

UNDER PEER REVIEW

(Fig. 2A), which is consistent with previous reports (9). These data indicate that ABL1 protein is overexpressed in a subset of human HCC.

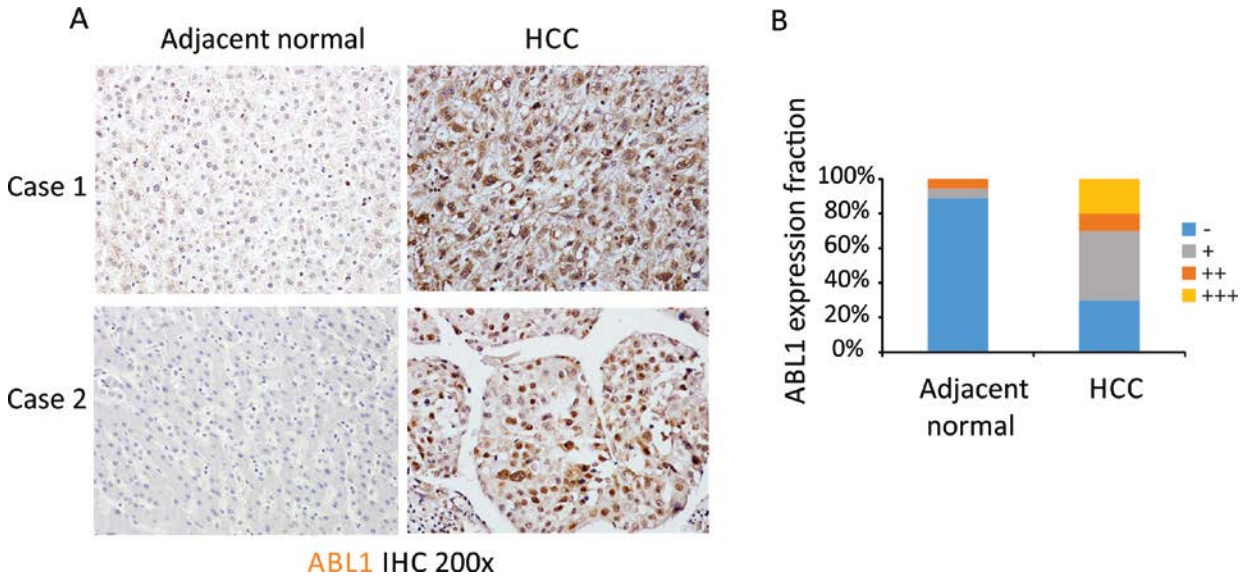

Figure 2. ABL1 protein is overexpressed in human HCC patients. (A) Representative photographs of IHC staining of ABL1 in human adjacent normal liver and HCC. **(B)** Quantification of expression of ABL1 in a human HCC TMA.

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, most of the p-ABL1 staining was observed in the HCC samples showing high expression of total ABL1 (86%, 22/26).

UNDER PEER REVIEW

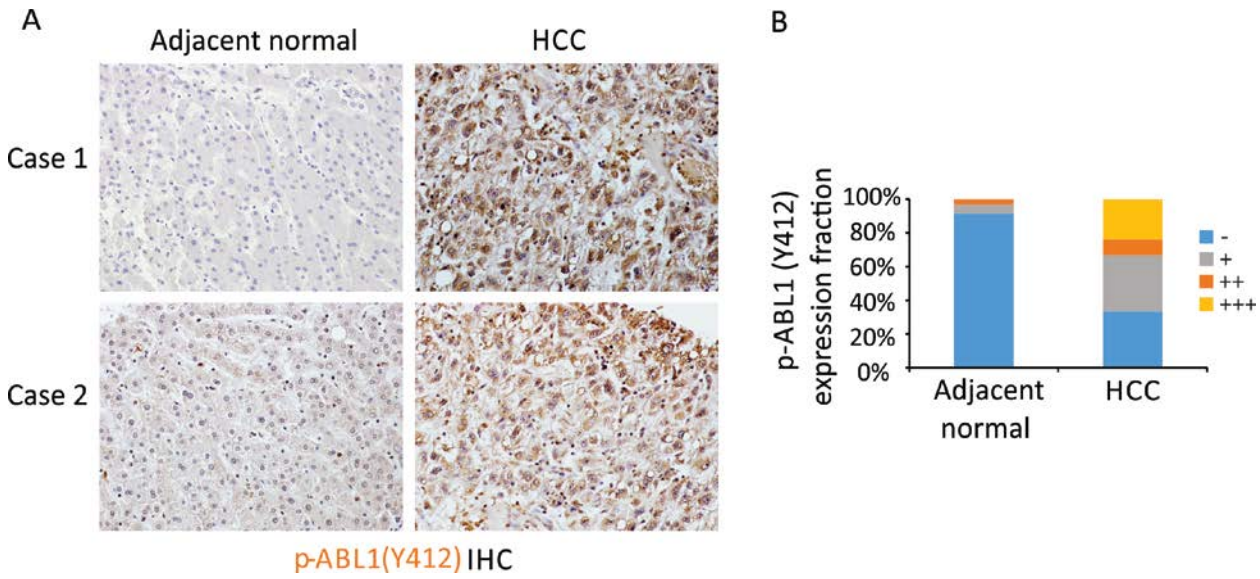

Figure 3. p-ABL1 expression is increased in human HCC patients. (A) Representative photographs of IHC staining of p-ABL1 (Y412) in human adjacent normal liver tissue and HCC. **(B)** Quantification of expression of p-ABL1 (Y412) in a human HCC TMA.

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 (Δ*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

UNDER PEER REVIEW

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.

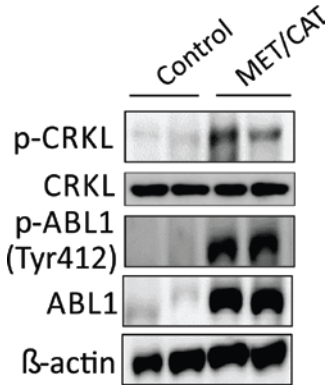

Figure 4. ABL1 is overexpressed and activated in MET/CAT-induced mouse HCC. Protein expressions of p-CRKL, CRKL, p-ABL1, ABL1 and β-actin in the livers of the mice treated with control and MET/CAT for 7 weeks.

4. DISCUSSION

The role of ABL1 in HCC remains unknown. In this study, we analyzed two separate database cohorts 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 HCC patients 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 samples

UNDER PEER REVIEW

with 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.

It has been shown that HGF/MET can directly bind to ABL and activate ABL in mouse mammary tumors and breast cancer cells (26). We found that ABL1 was activated in MET/CAT-induced tumors (Fig. 4). It is possible that MET might directly activate ABL1 in mouse hepatocytes. However, it is notable that total ABL1 expression was also increased in MET/CAT-induced tumors (Fig.4) while mRNA of ABL1 was not induced in these tumors (data not shown), suggesting a post-transcriptional or post-translational mechanism involved in ABL1 expression in this model. It is warranted to investigate the underlying mechanism in our future study.

In conclusion, our study shows that ABL1 is overexpressed and activated in a subset of human and mouse HCC. Because ABL1 plays an important role in cell survival, growth, adhesion, migration, and invasiveness, it is likely ABL1 may be involved in the pathogenesis of HCC. Our data provide a clinical basis to warrant further study of the role of ABL1 in the development of HCC, which is critical for potential therapeutic intervention by targeting ABL kinases clinically.

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.

UNDER PEER REVIEW

ETHICAL APPROVAL

It is not applicable.

UNDER PEER REVIEW

References

1. Bruix J, Sherman M, Practice Guidelines Committee AAftSoLD. Management of hepatocellular carcinoma. Hepatology. 2005 Nov;42(5):1208-36. PubMed PMID: 16250051.
2. Johnson PJ. Non-surgical treatment of hepatocellular carcinoma. HPB : the official journal of the International Hepato Pancreato Biliary Association. 2005;7(1):50-5. PubMed PMID: 18333161. Pubmed Central PMCID: 2023922.
3. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Haussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J, Group SIS. Sorafenib in advanced hepatocellular carcinoma. The New England journal of medicine. 2008 Jul 24;359(4):378-90. PubMed PMID: 18650514.
4. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. Nature reviews Cancer. 2006 Sep;6(9):674-87. PubMed PMID: 16929323.
5. Forner A, Gilabert M, Bruix J, Raoul JL. Treatment of intermediate-stage hepatocellular carcinoma. Nature reviews Clinical oncology. 2014 Sep;11(9):525-35. PubMed PMID: 25091611.
6. Finn RS. Treatment of Intermediate-Stage Hepatocellular Carcinoma. Gastroenterology & hepatology. 2015 Aug;11(8):545-7. PubMed PMID: 27118952. Pubmed Central PMCID: 4843044.
7. Shang N, Arteaga M, Zaidi A, Stauffer J, Cotler SJ, Zeleznik-Le N, Zhang J, Qiu W. FAK is required for c-Met/beta-catenin-driven hepatocarcinogenesis. Hepatology. 2014 Aug 28. PubMed PMID: 25163657.
8. Shang N, Arteaga M, Zaidi A, Colter S, Breslin P, Ding X, Kuo P, Nishimura M, Zhang J, Qiu W. FAK kinase activity is required for the progression of c-Met/beta-catenin-driven HCC. Gene expression. 2016 May 2. PubMed PMID: 27142958.
9. Greuber EK, Smith-Pearson P, Wang J, Pendergast AM. Role of ABL family kinases in cancer: from leukaemia to solid tumours. Nature reviews Cancer. 2013 Aug;13(8):559-71. PubMed PMID: 23842646. Pubmed Central PMCID: 3935732.
10. Colicelli J. ABL tyrosine kinases: evolution of function, regulation, and specificity. Science signaling. 2010 Sep 14;3(139):re6. PubMed PMID: 20841568. Pubmed Central PMCID: 2954126.
11. Ganguly SS, Fiore LS, Sims JT, Friend JW, Srinivasan D, Thacker MA, Cibull ML, Wang C, Novak M, Kaetzel DM, Plattner R. c-Abl and Arg are activated in human primary melanomas, promote melanoma cell invasion via distinct pathways, and drive metastatic progression. Oncogene. 2012 Apr 05;31(14):1804-16. PubMed PMID: 21892207. Pubmed Central PMCID: 3235241.
12. Srinivasan D, Sims JT, Plattner R. Aggressive breast cancer cells are dependent on activated Abl kinases for proliferation, anchorage-independent growth and survival. Oncogene. 2008 Feb 14;27(8):1095-105. PubMed PMID: 17700528.
13. Zhou S, Tang L, Wang H, Dai J, Zhang J, Shen L, Ng SW, Berkowitz RS. Overexpression of c-Abl predicts unfavorable outcome in epithelial ovarian cancer. Gynecologic oncology. 2013 Oct;131(1):69-76. PubMed PMID: 23820113.
14. Furlan A, Stagni V, Hussain A, Richelme S, Conti F, Prodosmo A, Destro A, Roncalli M, Barila D, Maina F. Abl interconnects oncogenic Met and p53 core pathways in cancer cells. Cell death and differentiation. 2011 Oct;18(10):1608-16. PubMed PMID: 21455220. Pubmed Central PMCID: 3172114.
15. Lin J, Sun T, Ji L, Deng W, Roth J, Minna J, Arlinghaus R. Oncogenic activation of c-Abl in non-small cell lung cancer cells lacking FUS1 expression: inhibition of c-Abl by the tumor suppressor gene product Fus1. Oncogene. 2007 Oct 25;26(49):6989-96. PubMed PMID: 17486070. Pubmed Central PMCID: 3457636.

UNDER PEER REVIEW

16. Xing QT, Qu CM, Wang G. Overexpression of Abl2 predicts poor prognosis in hepatocellular carcinomas and is associated with cancer cell migration and invasion. OncoTargets and therapy. 2014;7:881-5. PubMed PMID: 24940071. Pubmed Central PMCID: 4051810.
17. Arteaga M, Shang N, Ding X, Yong S, Cotler SJ, Denning MF, Shimamura T, Breslin P, Luscher B, Qiu W. Inhibition of SIRT2 suppresses hepatic fibrosis. American journal of physiology Gastrointestinal and liver physiology. 2016 Jun 1;310(11):G1155-68. PubMed PMID: 27125275. Pubmed Central PMCID: 4935480.
18. Shang N, Arteaga M, Chitsike L, Wang F, Viswakarma N, Breslin P, Qiu W. FAK deletion accelerates liver regeneration after two-thirds partial hepatectomy. Scientific reports. 2016 09/28/online;6:34316.
19. Qiu W, Liu H, Sebastini A, Sun Q, Wang H, Zhang L, Yu J. An apoptosis-independent role of SMAC in tumor suppression. Oncogene. 2013 May 09;32(19):2380-9. PubMed PMID: 22751125. Pubmed Central PMCID: 3751796.
20. Van Etten RA. Cycling, stressed-out and nervous: cellular functions of c-Abl. Trends in cell biology. 1999 May;9(5):179-86. PubMed PMID: 10322452.
21. Pluk H, Dorey K, Superti-Furga G. Autoinhibition of c-Abl. Cell. 2002 Jan 25;108(2):247-59. PubMed PMID: 11832214.
22. Tward AD, Jones KD, Yant S, Cheung ST, Fan ST, Chen X, Kay MA, Wang R, Bishop JM. Distinct pathways of genomic progression to benign and malignant tumors of the liver. Proceedings of the National Academy of Sciences of the United States of America. 2007 Sep 11;104(37):14771-6. PubMed PMID: 17785413. Pubmed Central PMCID: 1964540.
23. Stauffer JK, Scarzello AJ, Andersen JB, De Kluyver RL, Back TC, Weiss JM, Thorgeirsson SS, Wiltrout RH. Coactivation of AKT and beta-catenin in mice rapidly induces formation of lipogenic liver tumors. Cancer research. 2011 Apr 1;71(7):2718-27. PubMed PMID: 21324921. Pubmed Central PMCID: 3074499.
24. Patil MA, Lee SA, Macias E, Lam ET, Xu C, Jones KD, Ho C, Rodriguez-Puebla M, Chen X. Role of cyclin D1 as a mediator of c-Met- and beta-catenin-induced hepatocarcinogenesis. Cancer research. 2009 Jan 1;69(1):253-61. PubMed PMID: 19118010. Pubmed Central PMCID: 2628201.
25. de Jong R, ten Hoeve J, Heisterkamp N, Groffen J. Tyrosine 207 in CRKL is the BCR/ABL phosphorylation site. Oncogene. 1997 Feb 6;14(5):507-13. PubMed PMID: 9053848.
26. Li R, Knight JF, Park M, Pendergast AM. Abl Kinases Regulate HGF/Met Signaling Required for Epithelial Cell Scattering, Tubulogenesis and Motility. PloS one. 2015;10(5):e0124960. PubMed PMID: 25946048. Pubmed Central PMCID: 4422589.