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SHORT REPORT

Overexpression of Abl2 predicts poor prognosis in hepatocellular carcinomas and is associated with cancer cell migration and invasion

Quan-tai Xing
Chun-mei Qu
Gang Wang

Department of Infectious Diseases, Qilu Hospital of Shandong University, Jinan, People's Republic of China

Introduction: Abl2 nonreceptor tyrosine kinase (Arg, c-abl oncogene 2) has recently been identified as being recurrently amplified at DNA levels and overexpressed at mRNA levels in hepatocellular carcinomas (HCCs), and might be a potential oncogenic driver and therapeutic target for HCC.

Methods: In this study, we investigated the Abl2 expression in a series of HCC tumors by immunohistochemistry and further evaluated its clinicopathological and prognostic significance. We also performed an in vitro experiment to validate the effect of *Abl2* gene silencing on the migration and invasion abilities of human liver cancer HepG2 cells.

Results: It has been demonstrated that Abl2 was unregulated in 37.3% (28/75) of primary HCC tissues, and was significantly associated with a shorter overall survival time (P=0.0005). In addition, *Abl2* gene silencing in HepG2 cells significantly attenuated its migration and invasion abilities in vitro. We also found that the phosphorylation of metastasis-associated gene cortactin was markedly decreased by *Abl2* silencing.

Conclusion: We propose that Abl2 might be a potential candidate therapeutic target for HCCs and that targeted therapies against Abl2 in the treatment of HCCs deserve further investigation in the future.

Keywords: Abl2, Arg, HCC, metastasis, prognosis

Introduction

Hepatocellular carcinomas (HCCs) represent one of the most frequent cancer types worldwide, especially in the People's Republic of China.[1] Individuals with HCC showed highly variable clinical courses, and even after receiving standard treatment, there is still a noticeable proportion of patients with a poor prognosis. Therefore, exploration of novel prognostic or therapeutic targets for this devastating malignancy is urgently needed.

Somatic DNA alterations have been frequently reported in HCCs. Recurrent copy number aberrations would provide useful clues for identifying candidate oncogenic drivers or therapeutic targets for HCCs.[2] Recently, Wang et al[3] comprehensively characterized copy number aberrations and accompanying gene expression changes in tumors and cell lines from HCC patients, and identified a panel of oncogenic drivers, among which was Abl2 (Arg, c-abl oncogene 2), a nonreceptor tyrosine kinase, ranked as the top most frequently amplified and overexpressed gene, and thus may serve as a potential therapeutic target for the treatment of HCC.

Elevated Abl2 expression levels and activities have been detected in multiple cancers, such as colorectal cancers and melanoma;[4,5] however, its expression and

Correspondence: Quan-tai Xing
Department of Infectious Diseases, Qilu Hospital of Shandong University, 107 Wenhuaxi Road, Jinan 250012, People's Republic of China
Email qlyyxqt@163.com

clinical significance in HCC samples has not been investigated. Therefore, in this study, we assessed the expression of Abl2 protein in HCC tumors by immunohistochemistry and then evaluated its clinicopathological and prognostic significance. Moreover, the effect of silencing Abl2 on the migration and invasion abilities of HepG2 liver cancer cells in vitro was also observed.

Materials and methods

HCC samples

Ninety HCC tissues and their paired adjacent tissues from HCC patients who underwent curative resection from August 2006 to September 2010 were included in this study. According to the previous criteria,[6] 15 patients were excluded for lack of complete clinicopathological data or for patient's death caused by postsurgical complications within 2 months, which left 75 patients eligible for the following data analysis. Overall survival time was defined as time from operation to cancer-related death only. Median age was 55 years (range, 37–73 years). Follow-up was finished on September 2013. The median follow-up was 32 months (range, 4–79 months). The other clinical and pathological characteristics of these patients are seen in Table 1.

Immunohistochemistry

Abl2 protein expression was determined with the standard streptavidin peroxidase procedures according to the manufacturer's protocol (Zhongshan Golden Bridge

Biotechnology Co, Ltd, Beijing, People's Republic of China). Briefly, 4 μm thick paraffin-embedded tissue sections were deparaffinized and rehydrated. Endogenous peroxidase was quenched with methanol and 0.3% H_2O_2 for 30 minutes. Next, antigen retrieval was performed by microwave heating in 0.01 M sodium citrate. Immunostaining was detected with antihuman polyclonal antibody against Abl2 (Santa Cruz Biotechnology Inc., Dallas, TX, USA; at 1:250 dilution). Detection was performed, using diaminobenzidine as the chromogen. Nonspecific mouse immunoglobulin G was used as negative control. Immunostaining was defined as of high expression level if moderate or strong staining was observed in more than 10% of the cancer cells in HCC tissues. This method of quantification of immunostaining has been described and has been widely used previously.[7]

## Cell lines and silencing of *Abl2* gene expression with small interfering RNA

Human hepatoma cell lines HepG2 were obtained from the Shanghai Cell Bank of Chinese Academy of Sciences and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. Two different specific small interfering RNA (siRNA) duplexes for Alb2 and nonspecific control siRNA, which does not match any human genome sequence, were designed as previously described.[8,9] siRNAs transfection was conducted by use of the HiPerFect Transfection Reagent (Qiagen NV, Venlo, the Netherlands), according to the manufacturer's instructions. Forty-eight hours after the second transfection, cells were replated in fresh medium supplemented for further analysis. The gene silencing effects were evaluated by Western blot.

Transwell migration and invasion assays

After siRNAs transfection for 48 hours, Abl2 siRNA transfected cells and their controls were harvested and resuspended in medium containing 1% fetal bovine serum and then placed in the top chamber of transwell migration chambers at 5×10^4 cells/well. The lower chamber was filled with media supplemented with 10% fetal bovine serum. After 24 hours, unmigrated cells were removed from the upper surface of the transwell membrane, and the migrated cells on the lower membrane surface were fixed, stained with crystal violet, and counted under high-power magnification. The results were calculated as migration rates in relation to control cells. The procedure to perform transwell invasion assays was done under the same conditions as the migration assays except in matrigel-coated transwell.

Table 1 Association of Abl2 expression levels and clinicopathologic characteristics of hepatocellular carcinoma tumors

Characteristics	Number	Abl2 expression		*P*-value
		Low	High	
Sex				0.9896
Male	63	40	23	
Female	12	7	5	
Age, years				0.9021
≤60	57	35	22	
>60	18	12	6	
Cirrhosis				0.8805
Absence	53	33	20	
Presence	22	14	8	
Histological grade				0.2515
1	8	5	3	
2	55	37	18	
3	12	5	7	
American Joint Committee on Cancer stage				0.3249
I–II	39	27	12	
III–IV	36	20	16	
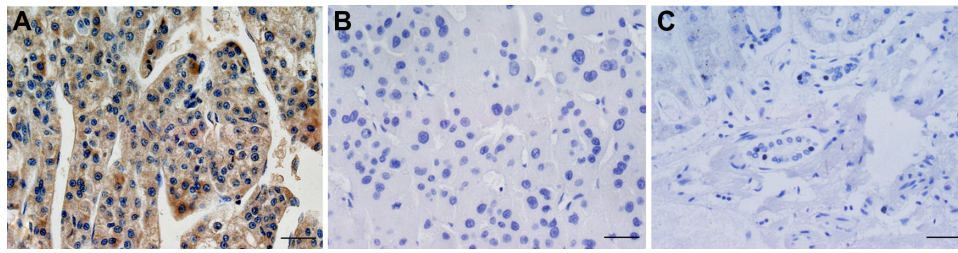
Figure 1 Representative images of immunoreactivity for Abl2 in hepatocellular carcinoma tumors (**A**, high expression; **B**, low expression) and adjacent tissues (**C**). (Scale bar, 50 μm).

Western blot

Cells were washed in cold PBS and lysed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, People's Republic of China). Fifty micrograms of whole-cell protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA). After blocking, membranes were first incubated with primary antibodies against Abl2 (1:2,000 dilution), cortactin (Santa Cruz Biotechnology Inc.; 1:2,000 dilution), and phospho-Cortactin (Tyr466; Santa Cruz Biotechnology Inc.; 1:1,500 dilution) overnight at 4°C and then were incubated with secondary antibodies conjugated to horseradish peroxidase. Bands were detected by chemiluminescence.

Statistics

The association between Abl2 expression and clinicopathologic characteristics was assessed by the chi-squared test with Yates correction. Overall survival of HCC patients with regard to Abl2 expression levels was estimated by the Kaplan–Meier method and log-rank test. A Cox proportional hazards regression model was adopted in the multivariate analysis. All statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). A P-value less than 0.05 was considered statistically significant. Migration and invasion rates were expressed as the mean ± standard deviation, and differences between groups were compared by Student's t-test.

Results

Representative immunohistochemical images of Abl2 in HCC tissues are seen in Figure 1. Normal adjacent liver tissues showed no or weak Abl2 immunostaining. Compared with normal adjacent liver tissues, Abl2 overexpressed significantly in HCC tumors. According to the established criteria, a total of 28 (37.3%) of 75 HCC tumors were classified into high Abl2 expression; the others were defined as a low-expression subgroup.

Next, we analyzed the association between Abl2 expression levels with clinicopathological characteristics and postoperative overall survival of HCC patients. As seen in Table 1, no significant association was observed between Abl2 expression levels and the common clinicopathological variables of HCC tumors. However, HCC cases with high Abl2 expression demonstrated a significantly shorter overall survival time than those with low Abl2 expression (log-rank test, P=0.0005; Figure 2). When all the prognostic clinical variables were included as covariables in a Cox proportional hazards model, high Abl2 expression remained as an independent prognostic predictor for HCC patients (hazard ratio, 1.9951; 95% confidence interval, 1.0627–3.7457; P=0.0325; Table 2).

Then, we also performed an in vitro analysis on the effect of Abl2 silencing on the cancer migration and invasion abilities of HepG2 human hepatoma cells. We found that downregulation of Abl2 gene expression significantly inhibited the migratory and invasive abilities of HCC cells in transwell assays compared with in control
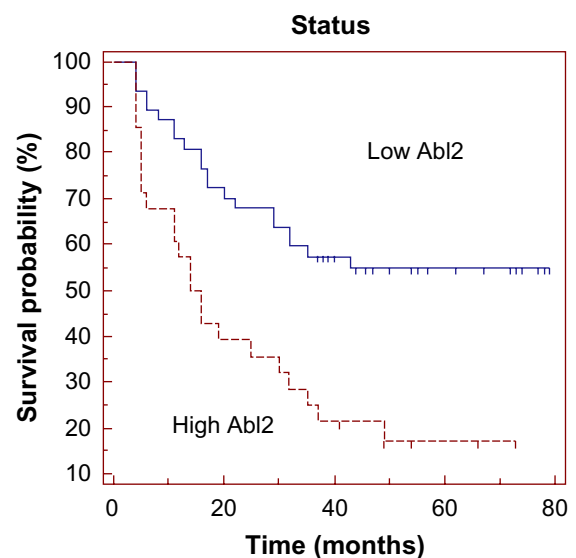
Figure 2 Overall survival curves in terms of different Abl2 expression levels in patients with hepatocellular carcinoma (P=0.0005, by log-rank test).

Table 2 Univariate and multivariate analysis of overall survival of hepatocellular carcinoma patients in terms of Abl2 protein expression levels and clinicopathologic parameters

Characteristics	Univariate		Multivariate	
	Hazard ratio (95% confidence interval)	P-value	Hazard ratio (95% confidence interval)	P-value
Age	0.6352 (0.2961–1.3625)	0.2462	0.9679 (0.4180–2.2412)	0.9395
Sex	0.5886 (0.2330–1.4867)	0.2647	0.7100 (0.2661–1.8947)	0.4963
Cirrhosis	0.7247 (0.3673–1.4300)	0.3556	1.3550 (0.7818–2.3485)	0.2815
Histological grade	1.9135 (1.0886–3.3633)	0.0249	0.9900 (0.4851–2.0207)	0.9781
American Joint Committee on Cancer stage	4.0462 (2.1107–7.7566)	<0.0001	3.0369 (1.5138–6.0925)	0.0019
Abl2	2.7142 (1.4990–4.9143)	0.0010	1.9951 (1.0627–3.7457)	0.0325

cells (Figure 3). To explore the mechanism by which Abl2 silencing inhibits the migration/invasive abilities of HepG2 cells, we observed its effect on the expression and activation of cortactin, a key modulator of cancer cell invasion. As seen in Figure 4, targeted silencing of the *Abl2* gene by siRNA also resulted in reduced phosphorylation of the cortactin in HepG2 cells.

Discussion

In this study, for the first time, we confirmed that Abl2 over-expressed in HCC tumors. Although no significant relation of Abl2 to clinicopathological parameters was observed, we demonstrated that Abl2 expression positively correlated to poor prognosis in HCCs and remained as an independent prognostic factor. Therefore, our findings indicated that highly Abl2-expressed tumors might represent a subset of HCCs with worse outcomes, which need more intensive or targeted treatment strategies.

Oncogenic tyrosine kinases have proved to be promising targets for the development of novel effective anticancer drugs.[10] Recently, many tyrosine kinase inhibitors against HCCs have entered clinical trials.[11] Abl2 is a member of the Abelson family of nonreceptor tyrosine protein kinases and plays an important role in cytoskeletal rearrangements through its C-terminal F-actin- and microtubule-binding sequences.[12] Our in vitro study demonstrated that silencing Abl2 attenuated the migration and invasion abilities of liver cancer cells. Similar phenomena could also be observed in breast cancer cells. Gil-Henn et al[13] demonstrated that Abl2 silencing significantly reduced breast cancer cell invasion, intravasation into blood vessels, and metastasis to lungs. Therefore, these findings indicated that the role of Abl2 in mediating invasion and metastasis might partly account for its close relationship with worse prognosis.

Accumulating evidence in other cancer cells indicates that Abl2 could phosphorylate protein cortactin, a master switch that activates invadopodium maturation and function, which in turn promotes cancerous matrix degradation and invasive cancer cells migration.[14,15] Cortactin is also a prognostic predictor for worse outcome for HCC tumors.[16]
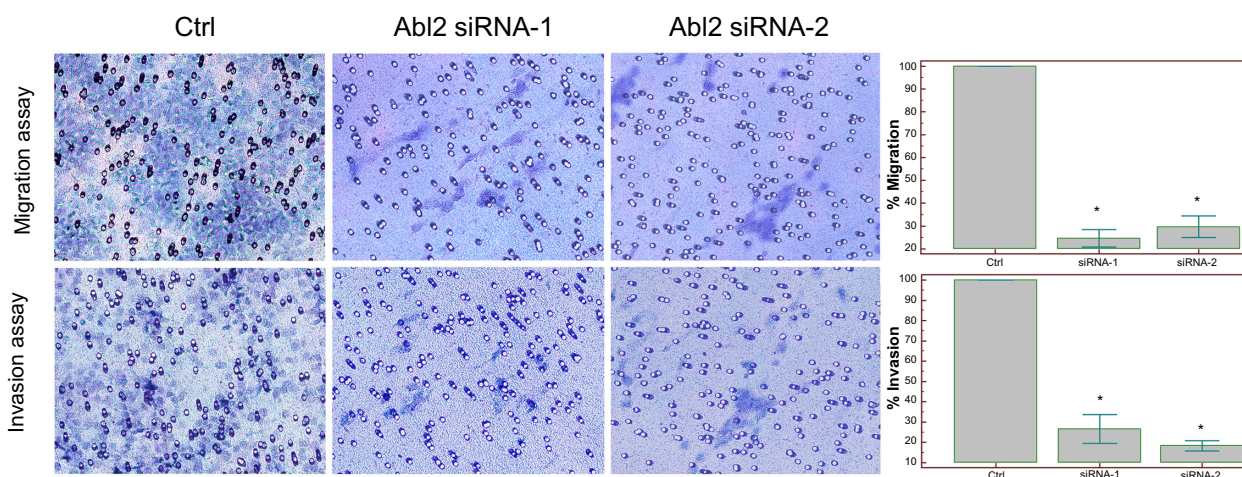
Figure 3 *Abl2* gene silencing significantly attenuated the migration and invasion abilities of human liver cancer HepG2 cells in vitro.
Note: *P<0.05.
Abbreviations: Ctrl, control; siRNA, small interfering RNA.
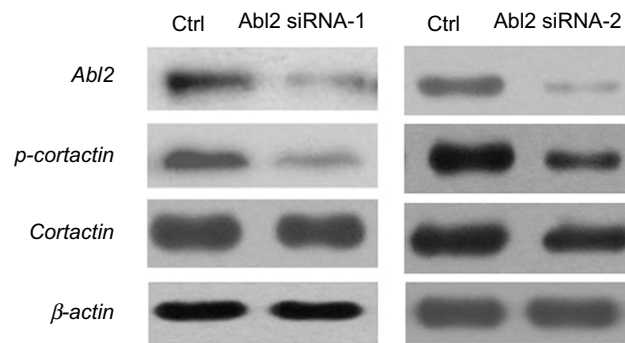
Figure 4 The phosphorylation of metastasis-associated gene *cortactin* was down-regulated by *Abl2* silencing in HepG2 cells.
Note: After *Abl2* silencing, the total expression of *cortactin* was not changed, while the phosphorylated form of *cortactin* was significantly downregulated.
Abbreviations: Ctrl, control; siRNA, small interfering RNA.

Our in vitro study further validated that the inhibitory effect of Abl2 silencing on the migration and invasion abilities is also accompanied by decreased cortactin phosphorylation in liver cancer cells.

Thus, on the basis of our and previous findings, we proposed that Abl2 might be a potential therapeutic target for HCC. A proportion of HCC patients with relatively worse outcome might benefit from Abl2-targeting inhibitors. According to our knowledge, this is the first study demonstrating a correlation between Alb2 and prognosis in HCCs. Furthermore, this present retrospective cohort study is limited to relatively small case series; therefore, further validation studies should be performed in larger samples from multicenters. Moreover, more-intensive studies targeting Abl2 in vitro and in vivo, using more liver cancer cell lines, are needed to explore the therapeutic potential in the future.

Conclusion

In conclusion, in this study, for the first time, we confirmed that Abl2 was overexpressed in HCC tissues and positively correlated with shorter survival of patients with HCC. We also found that silencing Abl2 could attenuate the migratory and invasive abilities through inhibiting the phosphorylation of cortactin in HepG2 cells. On the basis of these findings, we propose that Abl2 may be used as a novel candidate target for HCC and deserves further study.

Disclosure

The authors report no conflicts of interest in this work.

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