

Axl gene knockdown inhibits the metastasis properties of hepatocellular carcinoma via PI3K/Akt-PAK1 signal pathway

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Abstract The objective of this study is to clarify the possible role and mechanism of Axl in the tumorigenicity and metastasis process of hepatocellular carcinoma. The mRNA and protein expression levels of Axl in MHCC97-H and MHCC97-L cell lines were evaluated by real-time PCR and Western blot analysis. The key factor of phosphatidylinositol-3-kinase (PI3K)/Akt-p21-activated kinases-1 (PAK1) signaling pathway was studied after Axl expression was downregulated by shRNA. Finally, we analyzed the expression status of Axl protein expression in hepatocellular carcinoma tissues and its relationship with the prognosis of hepatocellular carcinoma. Axl was observed to be higher expressed in MHCC97-H cell lines compared to MHCC97-L cell lines. The downregulation of Axl in MHCC97-H cell lines resulted in the inhibition of the invasion ability of MHCC97-H cells both in vitro and in vivo. Interestingly, blocking PI3K/Akt signaling pathway by LY294002 or Akt siRNA could remarkably inhibit the PAK1 activation and cell invasion. Finally, the Axl protein expression was positively correlated with differentiation, lymph node metastasis, and clinical stage in patients with hepatocellular carcinoma patients (all $P < 0.01$). These findings suggest that Axl can also regulate the metastasis process of hepatocellular carcinoma and may serve as a new prognostic marker and therapeutic target for treating hepatocellular carcinoma metastasis.

Keywords Axl · PI3K/Akt · PAK1 · Metastasis · Hepatocellular carcinoma

Introduction

It was reported that receptor tyrosine kinases play a critical role in the control of some cellular processes, such as the cell cycle, cell metabolism, cell proliferation, cell differentiation, and cell invasion [1]. One subfamily is referred to as the TAM family, identified in 1991, comprising Tyro-3, Axl, and Mer. The primary ligand for TAM receptors is growth arrest-specific 6, a fairly large (75 kDa) vitamin K-dependent protein known to activate downstream signaling [2]. Adequate evidence supports the role of the Gas6/Axl system in driving cell growth and survival in normal and cancer cells [3].

Axl also called Ark Tyro7 and Ufo, initially identified as a transforming gene product [4], and Axl expression is reported to be upregulated in some human tumors [5]. Axl is ubiquitously expressed having been detected in a wide variety of organs and cells, including cell lines of epithelial, mesenchymal, and hematopoietic origins, as well as non-transformed cells [4]. Axl expression was reported to be associated with invasion and metastasis in some types of human cancers, including colon [6], esophageal [7], thyroid [8], breast [9], and lung carcinomas [10]. Axl activation is linked to several signal transduction pathways including Akt, MAP kinases, and NF- κ B [11]. However, the study investigating the expression and clinical implications of Axl in hepatocellular carcinoma (HCC) is still rare.

The phosphatidylinositol-3-kinase (PI3K)/Akt pathway is one of the core intracellular signaling pathways in the stimulation of growth factors. Expression of Akt or its phosphorylated form has been reported to be a significant indicator of prognosis in sarcoma [12], gastric cancer [13], pancreatic cancer [14], and breast cancer [15]. Previous study has

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reported that PI3K can activate p21-activated kinases-1 (PAK1) in renal proximal tubule cells independent of Akt activation [16]. Inhibition of PI3K/Akt activation abolished Axl-mediated PAK1 activation and cell invasion. So, Axl may promote cell invasion through the activation of PI3K/Akt-PAK1 pathway, but the mechanism of this pathway in HCC is still unknown. Targeting the PI3K/Akt-PAK1 pathway may be a useful tactic for the invasion of HCC.

In our previous study, it has shown that the expression of Axl could affect Hca-F cell proliferation, migration, invasion, which was also required for metastasis of HCC cells to lymph nodes, and it had different expression in the mouse HCC cell lines Hca-F and Hca-P cell lines [17, 18]. However, until now, little is known about the regulatory mechanism of Axl in the HCC metastasis. Therefore, we try to investigate the correlation between Axl and PI3K/Akt-PAK1 pathway and their role in the HCC metastasis.

Materials and methods

Cell culture and tissues

Human HCC cell lines MHCC97-H (high metastatic potential) and MHCC97-L (low metastatic potential) obtained from the Liver Cancer Institute Zhongshan Hospital, Fudan University, China were maintained in 90 % Roswell Park Memorial Institute (RPMI)-1640 (Gibco) and supplemented with antibiotics ($1\times$ penicillin/streptomycin 100 U/ml, Gibco) and 10 % fetal bovine serum (FBS) (Gibco). Cells were incubated in a humidified atmosphere containing 5 % CO₂ at 37 °C. Two cell clones of the same genetic background but with different metastatic potential were established from parental HCC cell line MHCC97. The parental cell line MHCC97 is a human HCC cell line performed on the animal model of human HCC LCI-D20. Compared with MHCC97-L, MHCC97-H had a high metastasis rate [19]. This study was done according to the ethical regulative of the institutional review board.

One hundred and thirty-seven paraffin-embedded HCC samples and 49 noncancerous paraffin-embedded normal liver samples were obtained from the General Surgery Department of the second affiliated hospital of Dalian medical University. In 137 HCC cases, there were 87 males and 50 females ranging in age from 14 to 79 years (median, 48 years). For the use of these clinical materials for research purposes, prior consents from the patients and approval from the Ethics Committees of the second affiliated hospital of Dalian Medical University were obtained, and all the procedures have been performed in compliance with the Helsinki Declaration. All specimens had confirmed pathological diagnosis and were staged according to the 2012 hepatocellular carcinomas staging system of the International Union against Cancer. Samples

were taken from mastectomy or wide local excision surgical specimens under the supervision of a pathologist. Fresh tissue samples were snap frozen in liquid nitrogen within 3 min of harvesting and stored at -80 °C.

Western blot analysis

Whole cell proteins were extracted from cells and HCC tissues (whole protein extraction kit KGP2100, KeyGEN). Protein concentration of the whole cells and tissues was measured with a bicinchoninic acid protein assay kit (KGPBCA, KeyGEN), and the protein was used for Western blot analysis.

The extracted protein was subjected to 10 % SDS-PAGE and then blotted onto polyvinylidene fluoride membranes (Pall Corporation). After blocking for 2 h with 5 % skimmed milk in PBS containing 0.1 % Tween 20 (PBST), we incubated the membranes with rabbit antihuman Axl polyclonal antibody (anti-Axl, ab72069, and Abcam), PI3K, Akt, PAK1 (4255, 2965, 3248-100, cell signaling), and the phosphorylation antibody of Akt, PAK1 (p-Akt, 9772, p-PAK1, Thr423, cell signaling) overnight in 5 % powdered skim milk buffer, washed thrice with PBS with 0.1 % Tween 20, and then incubated with secondary antibody anti-rabbit HRP (Santa Cruz Biotech Inc, 1/5,000 diluted). GAPDH polyclonal antibody (Santa Cruz Biotech Inc, 1/200 diluted) was used as controls. All blot analysis was performed with an ECL Western blotting kit (Amersham Biosciences, UK).

RNA isolation and real-time polymerase chain reaction analysis

Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Valencia, CA) and reverse transcribed into cDNA with QuantiTect Reverse Transcription Kit (QIAGEN, Valencia, CA) as described by the manufacturer. Quantitative real-time PCR was conducted with QuantiTect SYBR Green PCR Kit (QIAGEN, Valencia, CA) on 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA). RNA was converted to cDNA with the use of oligo d (T) 12–18 primers to preserve the relative mRNA profile and to produce a template suitable for PCR. The PCR primers Axl (F: 5-GGTGGCTGTGAAGACGATGA-3', R: 5-CTCAGATACTCCATGCCA-3') were used to confirm cDNA integrity and normalization of cDNA yields. In the PCR step, each 50 pmol of sense and antisense primer was used. Standard PCR amplification conditions consisted of a hot start at 94 °C for 5 min, followed by 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min for 30 cycles, with a final amplification at 72 °C for 10 min. The DNA was visualized using ethidium bromide staining, and band density was determined with a densitometer. The SYBR Green 1 Light Cycler reverse transcriptase PCR kit (Roche) was used for PCR amplification under conditions recommended by the manufacturer.

RNAi assay

RNAi assay was performed to silence Axl and Akt expression. MHCC97-H cells were incubated in appropriate antibiotic-free medium with 10 % FBS (Gibco), transferred to a 6-well tissue culture, and incubated at 37 °C, CO₂ incubator to obtain 60–80 % confluence. Thereafter, cells were transfected with shRNA targeting Axl (Axl shRNA, sc-29769-SH; Santa Cruz Biotechnology, Dalian, China) and Akt (Akt siRNA, sc-29195) or scrambled control shRNA (control shRNA; sc-37007, Santa Cruz Biotechnology) using Lipofectamine™2000 (#11668-019; Invitrogen) in serum-free OPTIMEM (GIBCO, Invitrogen) for 6 h at 37 °C, followed by incubation with complete medium for additional 24 h. When transient transfection was performed for 30 h, cells were harvested and followed with experiments. The cell transfection efficiency was 85 % and survival rate was 95 %, respectively.

In vitro cell invasion assays

Invasiveness of tumor cells was examined using 24-well transwell units (Corning, NY) with 8 mm pore size polycarbonate filter coated with Matrigel (BD Biosciences) to form a continuous thin layer. Cells (3×10^5) were harvested in serum-free medium containing 0.1 % BSA and added to the upper chamber. The lower chamber contained 500 mL 90 % RPMI 1640 and 10 % FBS. At the end of incubation, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab. The filters were fixed in methanol and stained with Wright–Giemsa. Cells invading the Matrigel that reached the lower surface of the filter were counted with light microscope at a magnification of $\times 400$. In migration assay, the upper chamber was not coated with Matrigel. Samples were acquired in triplicate and data expressed as the average cell number in five fields.

Tumorigenicity assay

Forty-eight 5-week-old male athymic nude mice were provided with sterilized food and water and equally divided into three groups. Approximately, 1×10^7 MHCC97-H cells (with or without Axl shRNA interference and control shRNA) were subcutaneously inoculated into the right flank of each nude mouse. When mice bear palpable tumors (about 3 week), mice were sacrificed and their tumors were isolated, weighed, and photographed. Experiments were repeated three times.

Immunohistochemistry

Immunohistochemical staining was conducted using formalin-fixed paraffin-embedded sections of tissues by the avidin-biotin-peroxidase complex (ABC) method. Four

micron sections of formalin-fixed paraffin-embedded tissues were cut with a microtome and dried overnight at 37 °C on a silicized slide (Dako, Carpinteria, CA, USA). Samples were deparaffinized in xylene at room temperature for 80 min and washed with a graded ethanol/water mixture and then with distilled water. The samples were soaked in a citrate buffer and then microwaved at 100 °C for 10 min. The following steps were used. Before addition of the primary antibodies, endogenous peroxidase activity was blocked by incubation in methanol containing 1 % H₂O₂ for 20 min, followed by 60 min incubation with normal donkey serum to reduce background staining. The primary antibodies, goat antihuman, Axl antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), were incubated at 4 °C for 8 h, followed by incubation with the biotinylated secondary antibodies (donkey anti-goat IgG; Santa Cruz Biotechnology) for 30 min and ABC complex for 30 min. The primary and secondary antibodies were used at 1:80 and 1:100 dilutions, respectively. The peroxidase binding sites were demonstrated by the diaminobenzidine method. A phosphate-buffered solution instead of the primary antibody was used in the protocols for negative controls.

Statistical analysis

The data were expressed as mean \pm SD, and Student's *t* test was used to determine the significance of differences in multiple comparisons. All data were analyzed with SPSS statistics software (version 13.0, Chicago, IL, USA) and a *P* value < 0.05 was considered statistically significant.

Results

Expression of Axl in MHCC97-H and MHCC97-L cell lines

We used real-time PCR and Western blot analysis to evaluate the expression status of Axl in mRNA and protein expression (Fig. 1). It was found that MHCC97-H cells got a higher Axl expression compared to the MHCC97-L cells.

Silence of Axl inhibits the invasive ability of MHCC97-H cells in vitro

Figure 1 showed that Axl was expressed higher in protein level in MHCC97-H cells compared to MHCC97-L cells (5.81-fold). We also downregulated the expression of Axl by RNAi approach in order to study the role of Axl in tumor invasion and metastasis. Axl expression was significantly reduced in Axl shRNA cells after 30 h transfection compared with both the control shRNA and the MHCC97-H cells (Fig. 2a). In addition, we also observed that Axl expression did not recover to the primary level until 14 days after shRNA transfection (data not shown).

Fig. 1 Expression levels of Axl are different in MHCC97-H and MHCC97-L cells revealed by real-time PCR and Western blot. **a** Axl gene expression was significantly higher in MHCC97-H cells than that in MHCC97-L cells. **b** Axl protein was abundant in MHCC97-H cells consistent with mRNA level. *Asterisk* indicates significant difference from the groups without an asterisk ($P < 0.01$)

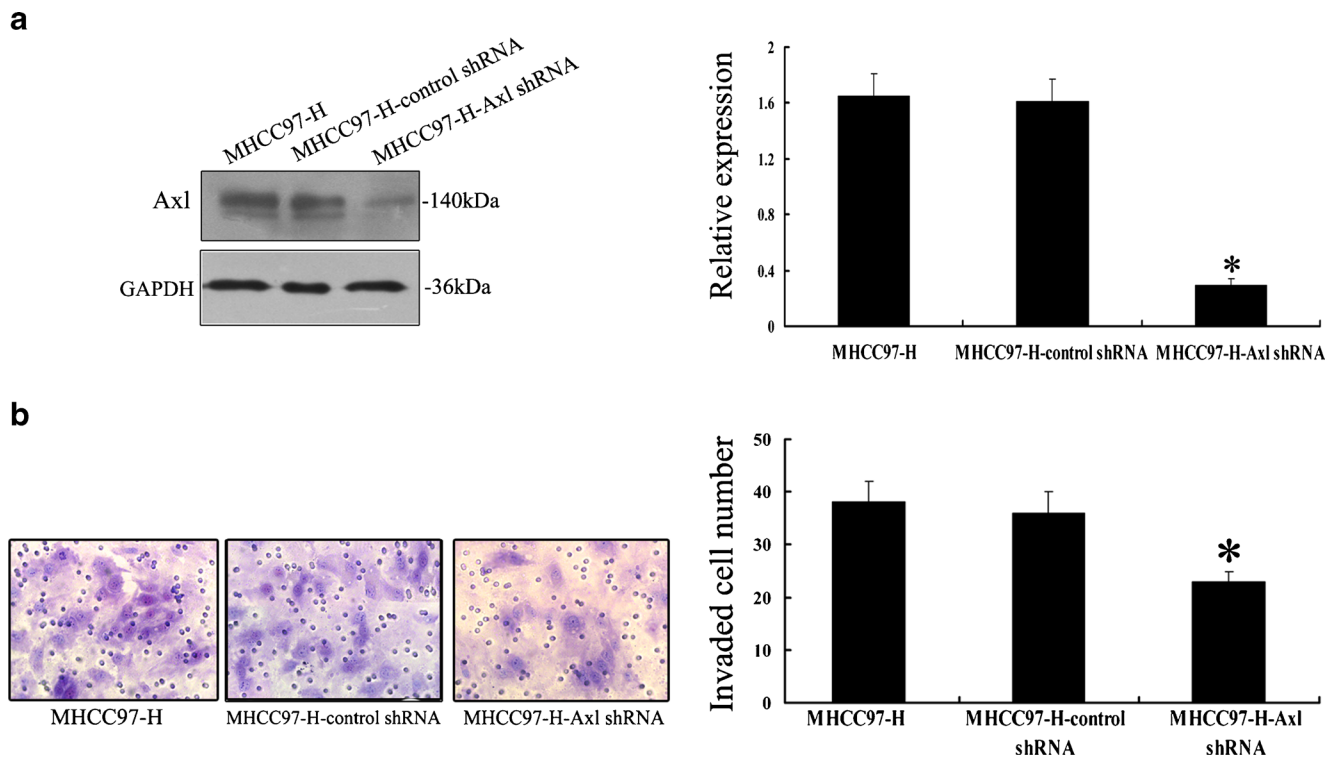
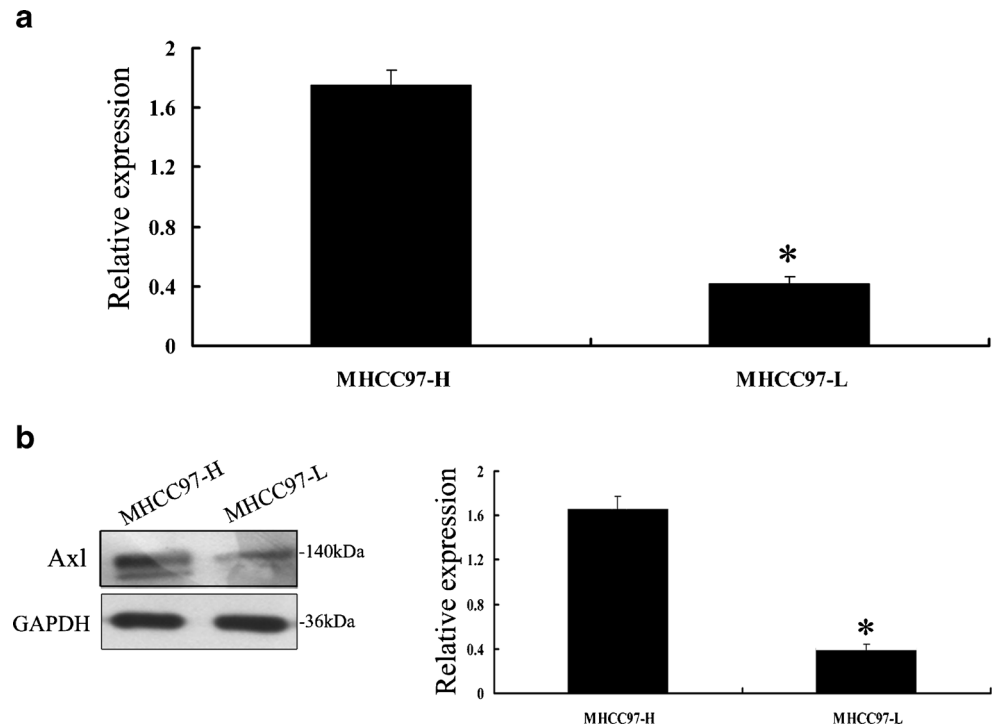


Fig. 2 Effects of Axl on the invasive and migration ability of MHCC97-L cells in vitro. **a** Silencing of Axl in MHCC97-L cells was analyzed by RNAi approach. After MHCC97-H cells were transfected with Axl shRNA for 30 h, Western blot analysis for Axl was assessed. GAPDH was also examined and served as controls for sample loading. Relative signal intensities of Axl protein levels were normalized against those of

GAPDH by LabWorks (TM ver4.6, UVP, BioImaging Systems) analysis, respectively ($*P < 0.05$). **b** In vitro ECMatrix gel analysis was performed. The average number of cells that invaded through the filter was counted. MHCC97-H cells were significantly more invasive ($*P < 0.05$) than the MHCC97-H-Axl shRNA cells and MHCC97-H-control shRNA cells. Data are the average \pm SD of triplicate determinants

We performed transwell invasion assay to further evaluate the invasion capability of Axl knockdown on tumor cells in vitro. It showed that the invasion capability of MHCC97-H cells transfected with control shRNA was not affected, but the invasion capability obviously decreased in those transfected with Axl shRNA (Fig. 2b).

Axl knockdown inhibits the tumorigenicity of MHCC97-H cells in vivo

MHCC97-H treated with and Axl shRNA cells were injected in the right flank of each nude mouse and with control shRNA as control. After 3 weeks of inoculation, a significant reduction in positive tumor in the Axl shRNA groups was observed compared with MHCC97-H and the control groups (Fig. 3a). The average weights ($n=16$) of tumors were 0.90 ± 0.07 , 1.50 ± 0.11 , and 1.50 ± 0.12 g in mice transplanted with Axl shRNA

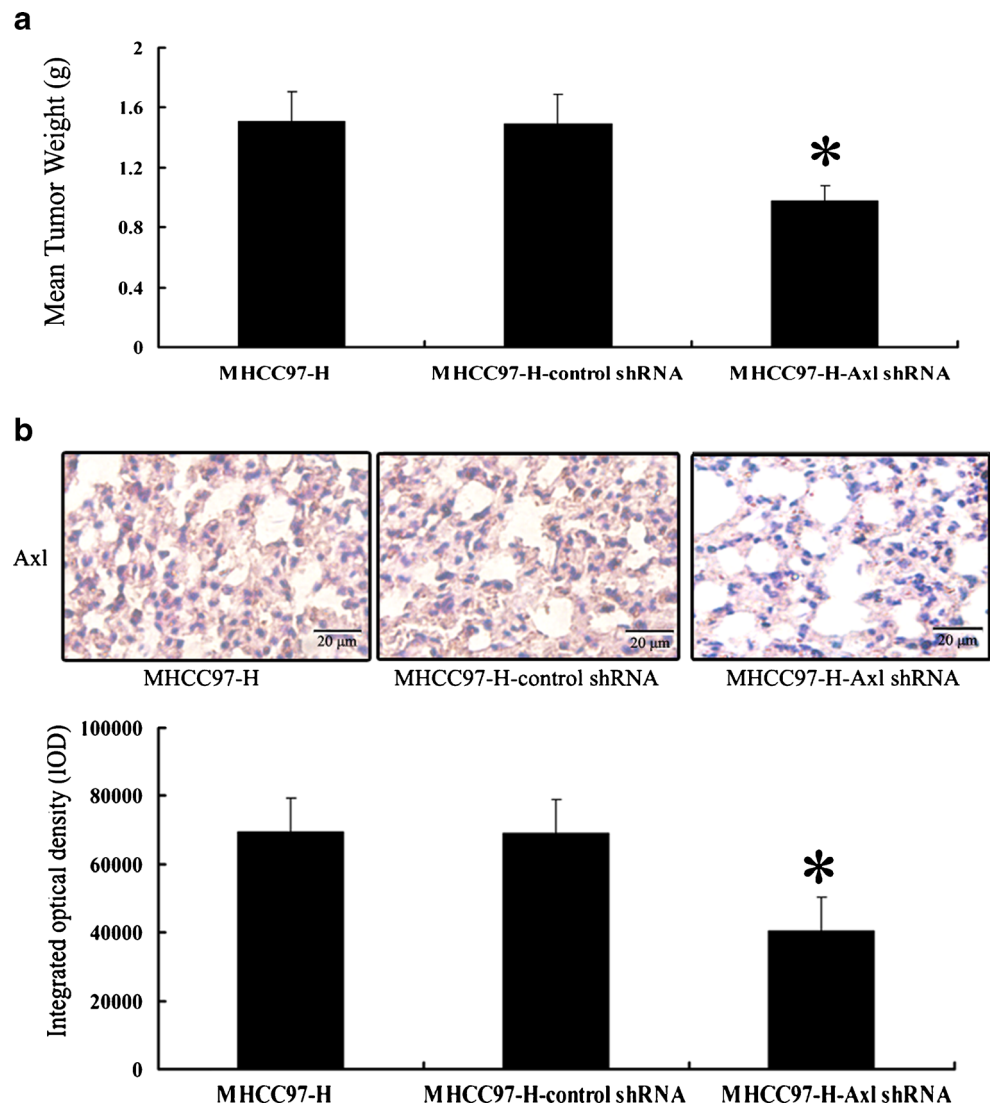
group, control shRNA, or MHCC97-H cells, respectively ($P<0.05$, Fig. 3a).

Then, we performed immunohistochemical studies of the expression of Axl in mice tumor tissues. It also revealed that Axl was highly expressed in tumors derived from control shRNA and parental MHCC97-H cells (Fig. 3b).

The relationship between Axl expression and PI3K/Akt-PAK1 signal pathway

We found that followed by the decreased expression level of Axl, the expression and activity of the PI3K/Akt pathway was inhibited. PI3K expression decreased the protein and phosphorylation levels of Akt; PAK1 were also downregulated after Axl silence (Fig. 4). PI3K, Akt, and PAK1 were downregulated at both protein level and activity in HCC cell Axl shRNA compared to those in HCC cells.

Fig. 3 Axl gene knockdown inhibits the tumorigenicity of MHCC97-H cells in vivo. 1×10^7 MHCC97-H cells (with or without Axl shRNA interference and control shRNA) were subcutaneously inoculated into the right flank of each nude mouse. When mice bearing palpable tumors (about 3 weeks), mice were sacrificed and their tumors were isolated, weighed, and photographed. **a** Significant reduction in mean tumor weight ($n=16$) of Axl gene knockdown was observed, as compared with MHCC97-H and control groups ($*P<0.05$ vs untreated MHCC97-H cells). **b** The protein levels of Axl were determined by immunohistochemistry staining in xenograft tumors derived from MHCC97-H, MHCC97-control shRNA, or MHCC97-H Axl shRNA cells. Differential protein levels of Axl were shown from independent experiments ($*P<0.05$ vs MHCC97-H cells; $*P<0.05$ vs MHCC97-H cells and MHCC97-H cells with control shRNA). (color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.)



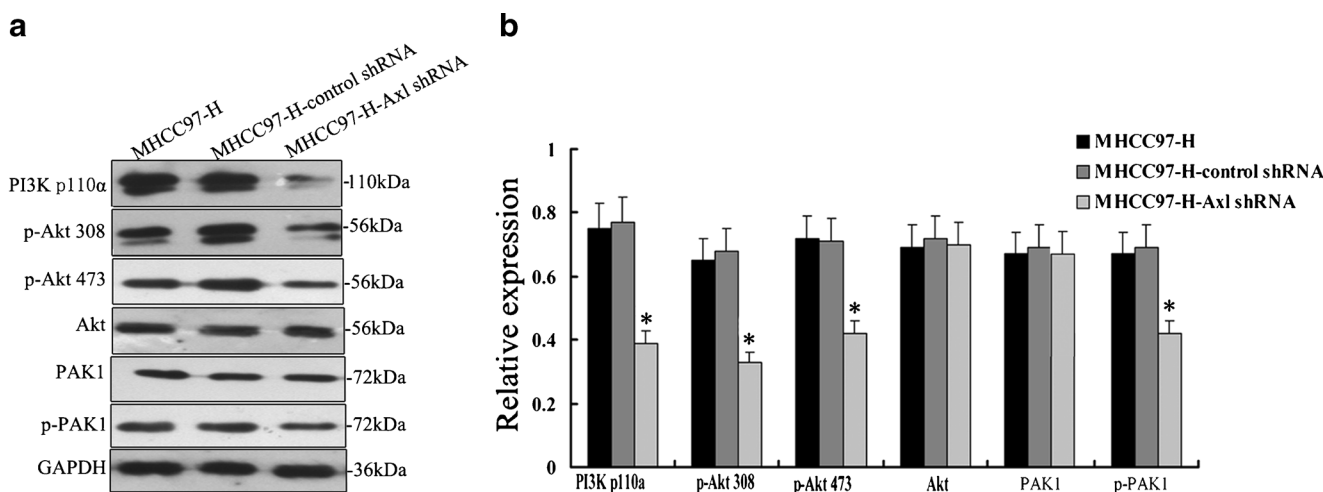


Fig. 4 Axl gene knockdown inhibits the activity of PI3K/Akt-PAK1 signal pathway. With Western blot analysis, the main signal molecules of PI3K/Akt-PAK1 signal pathway were found to be downregulated at

protein phosphorylation level (p-Akt, p-PAK1) in MHCC97-H cells with Axl shRNA, as compared with those in MHCC97-H cells (* $P < 0.05$ vs MHCC97-H cells and MHCC97-H cells with control shRNA)

The association between PI3K/Akt signal pathway and the invasion of MHCC97-H cells

After inhibited PI3K and Akt by LY294002 and Akt siRNA, the invasion ability was significantly inhibited in MHCC97 cells. Seventy to eighty percent of Akt protein expression reduced in protein level after 24–72 h treatment after inhibited Akt by siRNA. In addition, Akt inhibition remarkably reduced PAK1 phosphorylation (Fig. 5a). In transwell invasion assay, the invasive capability of MHCC97-H cells was not affected after transfected with control siRNA, but the invasive capability of MHCC97-H cells transfected with Akt siRNA obviously decreased in the transwell migration and invasion assay (Fig. 5b). Furthermore, we treated cells with LY294002, a PI3K inhibitor, and examined its effect on the invasion of MHCC97-H cells. Pretreatment with 20 $\mu\text{mol/L}$ LY294002 inhibited the MHCC97-H cells invasion (Fig. 5a).

Clinical implications of Axl protein expression in hepatocellular carcinoma

The Axl expression status was investigated in 137 paraffin-embedded primaries HCC with corresponding pericarcinomatous tissue samples by immunohistochemistry staining. Axl protein was stained at the nuclei of tumor cells. Finally, there was no significant association between Axl expression and age, gender, or distant metastasis in HCC patients ($P > 0.05$). Interestingly, we observed that the nuclear Axl expression was closely correlated with differentiation, lymph node metastasis, and clinical stage in patients with HCC ($P < 0.001$, $P < 0.001$, and $P < 0.001$, respectively). (Table 1)

Discussion

HCC is one of the most common malignancies with extreme poor prognosis. It was reported that in patients with symptomatic HCC, the 5-year survival rate is less than 5 % [20]. Due to the dismal prognosis of liver cancer with currently available therapies, there is an urgent need for new treatments based on a better understanding of the pathophysiologic and molecular properties of liver cancer, and now, our final goal was to identify genes which could contribute to differences in the ability to migration and invasion.

The present study showed that the receptor tyrosine kinase Axl was biologically relevant not only to HCC cells in vitro and animal models in vivo but also for human HCC. In our study, we have demonstrated that Axl was expressed predominantly in HCC cell lines and tissues. We found that Axl expression was higher in MHCC97-H cells, which had high metastasis potential than MHCC97-L cells with low lymphatic metastasis potential. These results were associated with the reported finding of Axl expression highly invasive lung adenocarcinoma cell lines compared with their less invasive counterparts [10]. Therefore, this might be evidence that Axl had an important effect on tumor metastasis.

The function of Axl in tumor progression including tumor growth, invasion, and migration was studied. shRNA was used to inhibit Axl expression in MHCC97-H cells, and we proved that silence of Axl could inhibit MHCC97-H cell invasion and tumorigenicity both in vitro and in vivo. The involvement of Axl in promoting cancer cell migration and invasion also has been demonstrated in vitro [21–24]. These findings supported that Axl plays an important role in the metastatic progression of various cancer types.

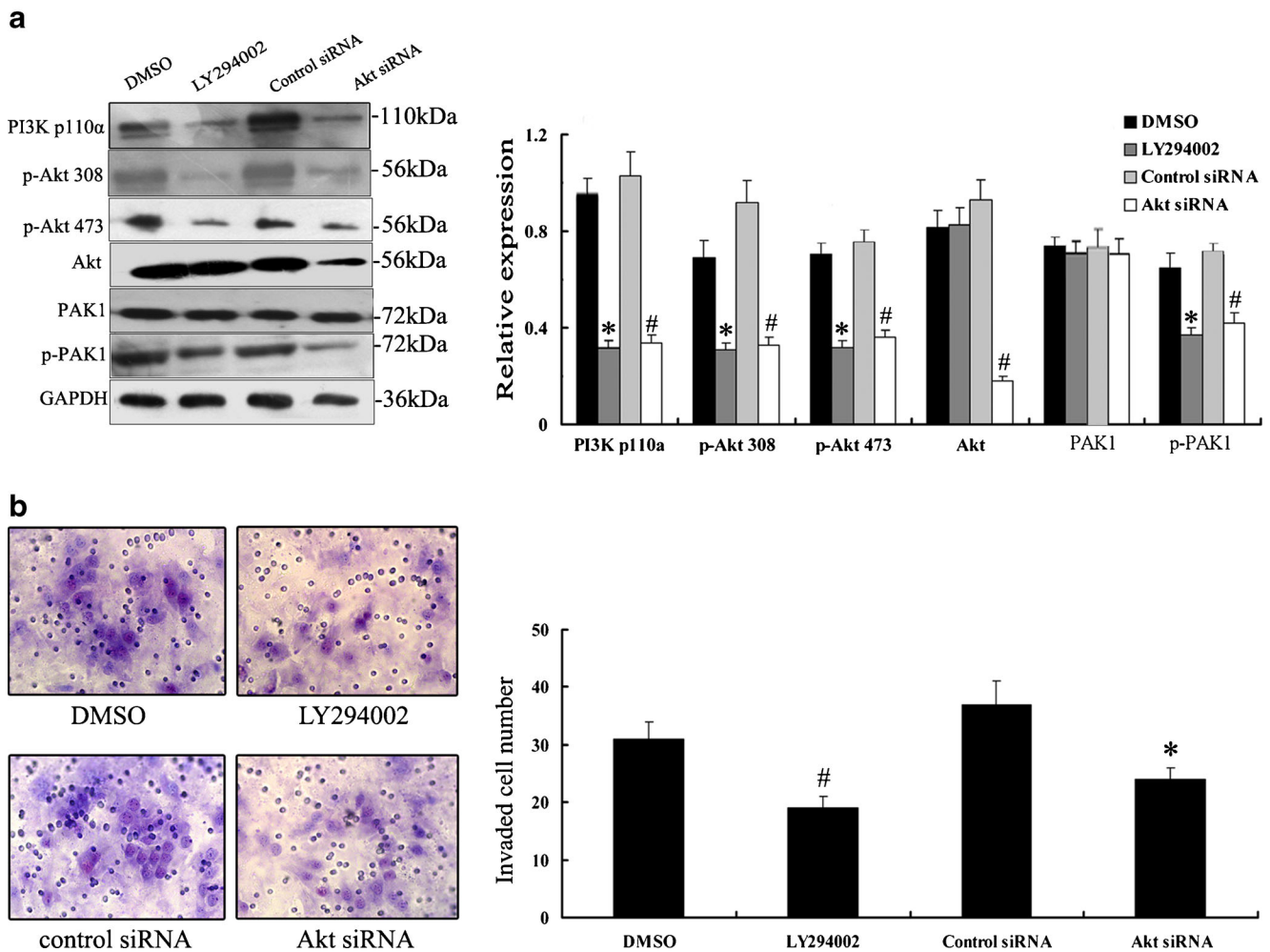


Fig. 5 Axl-induced PAK1 activation and cell migration are mediated by the PI3K/Akt signaling pathway. **a** Axl-mediated activation of PAK1 requires PI3K. Cells were treated with 20 μ mol/L LY294002 and Akt siRNA (T308A/S473A) and then subjected to Akt and PAK1 analysis. The phosphorylation levels of Akt and PAK1 were found to be downregulated in MHCC97-H cells treated with LY294002 and Akt siRNA (T308 or S473) compared with those in MHCC97-H cells and MHCC97-H cells treated with control Akt siRNA. **b** Inhibitor of PI3K or overexpression of

DN Akt inhibited Axl-mediated cell migration. Cells were pretreated with 20 μ mol/L LY294002 for 30 min or transiently transfected with DN Akt (T308A/S473A) and then in vitro ECMatrix gel analysis was performed. The average number of cells that invaded through the filter was counted. MHCC97-H-Akt siRNA and MHCC97-H-LY294002 cells were significantly less invasive ($*P < 0.05$) than the MHCC97-H and MHCC97-H-control Akt siRNA cells. Data are the average \pm SD of triplicate determinants

It is of interest to explore the molecular mechanism of Axl gene playing roles in physiological process and disease development. Axl has been demonstrated to be involved in several signal transduction pathways. The PI3K/Akt pathway is one of the core intracellular signaling pathways. In this study, we evaluated the correlation of the Axl-mediated PI3K/Akt signaling pathway with cell invasion. We demonstrated that MHCC97-H cells presented higher PI3K/Akt activity than the MHCC97-L cells, which was in accordance with the invasion phenotype. Altered expression of Axl markedly modulated the activity of PI3K/Akt pathway in human HCC cell lines. In addition, inhibition of the PI3K/Akt pathway with Akt-specific inhibitor LY294002 or Akt gene silencing

by siRNA pretreatment decreased the invasion of MHCC97-H cells. These results indicated that Axl-modulated HCC cell invasion was, at least in part, PI3K/Akt-dependent.

Although we have demonstrated that Axl was overexpressed in HCC cell lines and was associated with cancer cell invasion and tumorigenicity, its clinical feature was still unknown and should be detected. In the present study, we utilized immunohistochemistry to evaluate protein expression of Axl in HCC and further analyze its protein expression in clinicopathologically characterized 137 HCC cases. Our study suggested that cancer tissues had a higher expression level compared with peritumoral tissues. Subsequently, we analyzed the correlation of Axl expression with

Table 1 Correlation between the clinicopathologic characteristics and expression of Axl protein in hepatocellular carcinoma

Characteristics	n	Axl (%)		P value
		Axl (high)	Axl (low)	
Group				
Cancer tissue	137	73 (53.3 %)	64 (46.7 %)	0.011
Pericarcinomatous tissue	49	16 (32.7 %)	33 (67.3 %)	
Gender				
Male	87	51 (58.6 %)	36 (41.4 %)	0.112
Female	50	22 (44 %)	28 (56 %)	
Age (years)				
≥50	69	36 (52.2 %)	33 (47.8 %)	0.865
<50	68	37 (54.4 %)	31 (45.6 %)	
Differentiation				
E1, E2, or Well	98	42 (42.9 %)	56 (57.1 %)	0.001
E3, E4, or poorly	39	31 (79.5 %)	8 (20.5 %)	
Lymph node metastasis				
Absent	64	22 (34.4 %)	42 (65.6 %)	0.001
Present	73	51 (69.9 %)	22 (30.1 %)	
Distant metastasis				
Yes	33	22 (66.7 %)	11 (33.3 %)	0.109
No	104	51 (49 %)	53 (51 %)	
Clinical stage				
I~II	56	11 (19.6 %)	45 (80.4 %)	0.001
III~IV	81	62 (76.5 %)	19 (23.5 %)	

clinicopathologic features in HCC. Our results indicated that significantly increased protein expression of Axl closely associated with differentiation ($P<0.001$), lymph node metastasis ($P<0.001$), and clinical stage ($P<0.001$) in patients with HCC, which hinted that Axl as a growth factor might play an important role in HCC genesis and progression rather than distant metastasis ($P=0.109$). Axl has also been reported to be expressed 10-fold more in colon cancer metastasis than in other normal and malignant tissues [6]. These results suggested that high expression of Axl might be associated with tumor metastasis and Axl might be initially identified as a protein encoded by a transforming gene from primary human HCC. Further study is needed to determine whether Axl will be useful as a therapeutic target in a subgroup of HCC.

In conclusion, our results indicated that Axl played an important role in association with HCC cells invasion via modulating the PI3K/Akt signaling pathway. We also found that elevated expression of Axl was not only shown in HCC tissue compared with noncancerous liver tissues but also closely was associated with differentiation, lymph node metastasis, and clinical stage in patients with HCC. These findings were of potential pathophysiological importance for understanding the integration of migration-related signaling and provided a basis for designing future therapeutic strategy for blocking HCC metastasis in patients.

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Conflicts of interest None

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