The cell adhesion molecule L1 promotes gallbladder carcinoma progression *in vitro* and *in vivo*

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Abstract. Recent studies have demonstrated that the cell adhesion molecule, L1, is expressed in several malignant tumor types and its expression correlates with tumor progression and metastasis. However, the role of L1 in gallbladder carcinoma (GBC) remains unclear. Here, we demonstrate that L1 is expressed in GBC cells and plays an important role in the growth, motility, invasiveness, and adhesiveness of GBC cells. Specific depletion or overexpression of L1 in the GBC cell lines JCRB1033 and SNU-308, respectively, was achieved by lentivirus-mediated transduction and expression of an L1 mRNA-specific short hairpin RNA or full-length human L1. Stable depletion of L1 led to a significant decrease in GBC cell proliferation, migration and invasion, as well as decreased intracellular signaling through AKT and FAK. Overexpression of L1 in GBC cells enhanced these cellular activities. In a GBC xenograft nude mouse model, suppression of L1 markedly reduced tumor growth and increased the survival of tumor-bearing mice whereas L1 overexpression stimulated tumorigenicity. Taken together, these results suggest that L1 plays a crucial role in GBC progression and may be a novel therapeutic target in GBC treatment.

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Introduction

Gallbladder carcinoma (GBC) is the most common malignancy of the biliary tract. Although occurrence is rare compared to other gastrointestinal tract neoplasms such as gastric and colorectal cancers, GBC has a distinctly higher incidence in certain demographic groups and geographic areas (1,2). The prevalence of GBC in China, Thailand and Northern India is much higher than in the United States and Europe (3). Surgical resection is currently the most effective and potentially curative treatment for GBC. However, many patients present late in the course of the disease when surgical intervention is no longer effective; most patients with advanced GBC experience frequent recurrence after surgery. Therefore, the overall survival rate remains quite poor (4). Although the most important risk factor for the development of GBC is cholelithiasis (up to 95% of GBCs are associated with gallstones) (5), the molecular mechanisms involved in early carcinogenesis and the factors affecting disease progression remain unclear. Therefore, the identification of factors crucial for GBC progression is necessary for development of new therapeutic strategies.

L1 is a transmembrane glycoprotein of 200-220 kDa that belongs to the immunoglobulin superfamily of cell adhesion molecules. It consists of six immunoglobulin-like domains and five fibronectin type III repeats in the extracellular region, a single-pass transmembrane domain and a cytoplasmic tail (6). L1 was originally described as a neural cell adhesion molecule and has been shown to initiate a variety of dynamic motile processes, including cerebellar cell migration and neurite extension in the central nervous system (7,8). L1 is also expressed in other cell types such as endothelial cells, certain epithelial cells, reticular fibroblasts, and cells of lymphoid and myelomonocytic origin (9,10). Recent reports have shown that L1 is also expressed in several types of malignant tumors including gliomas, melanomas, endometrial and ovarian carcinomas, colorectal carcinomas, renal carcinomas, neuroendocrine carcinomas and extrahepatic cholangiocarcinomas (11-13) and that its expression correlates

with tumor progression and metastasis (11,14). However, the exact role of L1 in GBC cells has not been previously investigated.

The mechanism by which L1 contributes to tumor progression is not clearly established, but ligand binding likely plays an essential role in L1 signaling. Recent studies demonstrated that the cytoplasmic domain of L1 plays an important role in carcinogenesis via activation of extracellular signal-regulated kinase (ERK) and ERK-regulated expression of genes that contribute to cell growth, motility and invasion of tumor cells (14-16). Moreover, activation of ERK, AKT and focal adhesion kinase (FAK) are associated with L1-mediated survival of tumor cells (17,18). In addition, L1 mediates homophilic binding between cells and forms heterophilic interactions with various extracellular matrix proteins and their receptors (integrins), implying that L1 plays a role in promoting cancer and metastasis by mediating interactions between cells and the extracellular matrix (19,20). An association between the receptor tyrosine kinase family and L1 function was also reported (21). Although a direct interaction between full-length L1 and fibroblast growth factor receptor has not been demonstrated, activation of this receptor is apparently linked to L1-mediated cell proliferation and invasion in ovarian cancer (22). It was shown that the extracellular domain of L1 that is shed from the cell surface via proteolytic cleavage stimulates the migration and survival of tumor cells through autocrine/paracrine binding to integrins (23,24). This suggests that suppression of L1 interferes with various cellular functions.

In the present study, we investigated the functional role of L1 in GBC progression and clearly showed that suppression of endogenous L1 levels in GBC cells by expression of a short hairpin RNA (shRNA) specific for L1 mRNA decreased cell proliferation, migration and invasion as well as intracellular AKT and FAK signaling, whereas overexpression of L1 enhanced these cellular activities. In addition, L1 shRNA markedly reduced tumor growth in nude mice bearing a GBC xenograft whereas L1 overexpression conferred xenograft tumorigenicity. Our results suggest that L1 plays an important role in tumor progression of GBC and may serve as a potential target for development of GBC therapies.

Materials and methods

Cell lines and cell culture. JCRB1033 cells established from a moderately differentiated gallbladder adenocarcinoma (25) and SNU-308 cells (26) established from a well-differentiated gallbladder adenocarcinoma were grown in Williams' E medium (Invitrogen, Carlsbad, CA, USA) and RPMI-1640 medium (Invitrogen) supplemented with 10% FBS, respectively. All cells were maintained at 37˚C under 5% CO_2 and 95% relative humidity.

Short hairpin RNA targeting the L1 mRNA and L1 overexpression in GBC cells. Knockdown or overexpression of L1 in GBC cells was achieved through the use of lentivirus-mediated transduction of an L1 mRNA-specific short hairpin RNA (shRNA) interference using Mission RNAi system clones (Sigma-Aldrich, St. Louis, MO, USA) or a full-length human L1 (27) subcloned into a lentiviral vector (Macrogen, Daejeon, Korea), respectively. To generate stable transfectant, lentiviral vectors and packaging vectors were cotransfected into HEK293T cells with packaging vectors using Lipofectamine, according to the manufacturer's instructions (Invitrogen). The next day, virus harvested from the supernatant was added to JCRB1033 or SNU-308 cells along with 5 μg/ml polybrene. After 24 h, the medium was removed and replaced with fresh medium containing 1.2 μg/ml puromycin. Puromycin-resistant clones were selected by culture for 1 week in the presence of puromycin. L1 expression level was analyzed by RT-PCR and Western blotting.

Cell proliferation assay. GBC cells ($2x10^5$/ml) were seeded in 6-well dishes in 2 ml medium containing 1 or 3% FBS. After 72 h, viable cells were counted in a cell counter (Innovatis AG, Bielefeld, Germany). Statistical significance of the data was evaluated using the Student's t-test.

[^{3}H]thymidine incorporation assay. GBC cells were seeded at a density of $2x10^4$ cells/well in 24-well plates in medium containing 1% FBS. After 36 h, the cells were incubated with 0.5 μCi/ml of [^{3}H]thymidine for 6 h. High molecular weight DNAs were precipitated using 10% trichloroacetic acid at 4˚C for 30 min. The cells were washed twice with ice-cold H_2O and were solubilized in 0.2 N NaOH/0.1% SDS. ^{3}H-radioactivity was determined by liquid scintillation counter.

Migration and invasion assays. Migration and invasion of GB cells was performed using Transwell (Corning Costar, Cambridge, MA) with 6.5 mm diameter polycarbonate filters (8 μm pore size). Briefly, the lower surface of the filter was coated with 10 μg of gelatin for migration assay and the upper side was coated with 25 μg reconstituted basement membrane substance for invasion assay (Matrigel; BD Biosciences). The fresh medium containing 2% FBS (JCRB1033) or 5% FBS (SNU-308) was placed in the lower wells. GB cells were incubated for 24 h in medium containing 1% FBS and trypsinized and suspended at a final concentration of $1x10^6$ cells/ml in medium containing 1% FBS. The cell suspension (100 μl) was loaded into each of the upper wells. The chamber was incubated at 37˚C for 18 h (migration) or 24 h (invasion). Cells were fixed and stained with H&E. Non-migrating cells on the upper surface of the filter were removed by wiping with a cotton swab. Chemotaxis was quantified by counting the cells that migrated to the lower side of the filter with an optical microscope (x200). Eight random fields were counted for each assay.

Soft agar colony forming assay. Anchorage-independent growth assays were performed using the CytoSelect™ 96-well Cell Transformation assay kit (Cell Biolabs, San Diego, CA, USA). Briefly, JCRB1033 cells ($5x10^4$) or SNU-308 cells ($1x10^5$) were plated in DMEM containing 10% FBS in a cell suspension agar matrix between layers of base agar matrix. After 2 weeks, the agar matrix was solubilized and the cells were stained with MTT solution. Absorbance at 570 nm was measured using a microplate reader.

Cell adhesion assay. Ninty-six-well culture plates were coated with purified collagen, laminin, fibronectin or vitronectin at a concentration of 2 μg/ml each and incubated at 37˚C for 18 h. Wells were washed with PBS, incubated with $2x10^4$ cells in

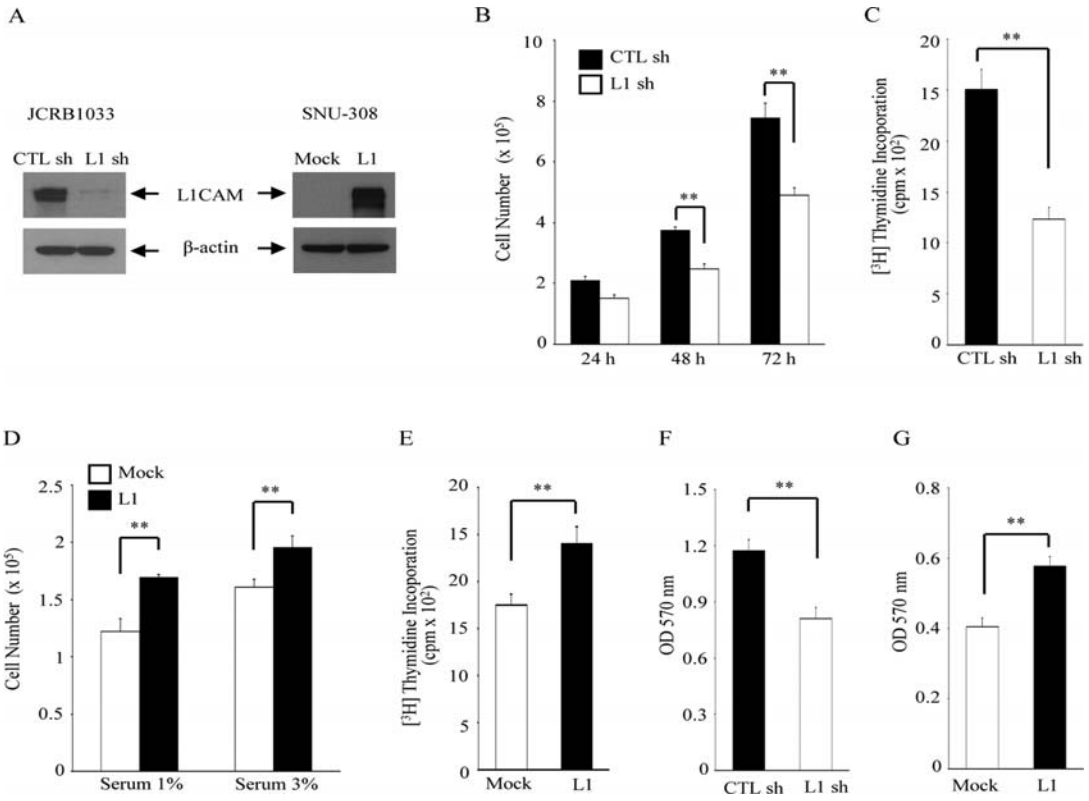

Figure 1. L1 regulates the growth of GBC cells. (A) JCRB1033 or SNU-308 cells were stably transfected with L1 shRNA or full-length human L1 expression plasmid, respectively, using lentivirus as described in Materials and methods. The expression of L1 was determined by Western blot analysis using an antibody against L1. *CTL* indicates cells transfected with control shRNA. (B and D) Stable transfectants were seeded at a density of $2x10^5$ cells/well in 6-well dishes containing 2 ml medium supplemented with 1% (B and D) or 3% (D) FBS. After incubating for the indicated times (B) or for 72 h (D), viable cells were counted in a cell counter. (C and E) A [^{3}H]thymidine incorporation assay was performed in JCRB1033 (C) or SNU-308 (E) stable transfectants as described in Materials and methods. (F and G) *In vitro* analysis of the clonogenic capacity was performed in JCRB1033 (F) or SNU-308 (G) stable transfectants as described in Materials and methods. Three independent experiments were performed in duplicate. Data are expressed as the mean ± SD [**P<0.01 vs. control (CTL) sh (B, C and F) or vs. mock (D, E and G)].

serum-free RPMI-1640 supplemented with 1% bovine serum albumin at 37˚C for 30 min, and then washed three times to remove unbound cells. The attached cells were measured by a WST1 assay. Briefly, the cells were incubated with WST1 reagent (Roche Diagnostics, Basel, Switzerland) at 37˚C for 30 min, and the absorbance was measured at 450 nm using a microplate reader. Data reflect the means of triplicates ± SD.

Western blot analysis. Cell lysates or immunoprecipitates from cell lysates were subjected to SDS-PAGE and then transferred to PVDF membranes (Chemicon, Temecula, CA, USA). The membranes were incubated with the indicated primary antibody followed by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the immunoreactive polypeptides were visualized using a chemiluminescent substrate (GE Life Sciences, Piscataway, NJ, USA).

Nude mice xenograft assays. Nude mice (6 weeks old) were obtained from the Charles River Laboratories. Mice were housed under specific pathogen-free conditions and were treated in accordance with the guidelines of the Animal Care Committee at the Korea Research Institute of Bioscience and Biotechnology. JCRB1033 cells ($5x10^6$) or SNU-308 cells ($1x10^7$) were inoculated subcutaneously into the right flank of each mouse. Tumor growth was monitored at 3-7 day intervals by measuring the length and width of the tumor with a caliper and calculating tumor volume using the following formula: volume = $0.523Lw^2$, where L is length and w is width. Once tumor volumes reached >2000 mm^3, mice were monitored for 30 days to determine percent survival. Percent survival was plotted against time after injection (Kaplan-Meier survival function) and compared using log-rank test analysis (StatView software; Abacus Concepts Inc., Piscataway, NJ, USA). Differences in survival were considered statistically significant when P-values were <0.05.

Statistical analysis. Data represent the mean ± SD, and statistical comparisons between groups were performed using one-way ANOVA followed by Student's t-test. A value of P<0.05 was considered significant.

Results

L1 is involved in proliferation of GBC cells. To investigate whether L1 plays a crucial role in GBC tumor progression, we generated GBC cell clones with stable knockdown or overexpression of L1. For these experiments, we chose two GBC cell lines; JCRB1033 expresses high levels of L1 and was used for knockdown studies and SNU-308 expresses low levels of L1 and was used for overexpression studies. Specific depletion or overexpression of L1 in these cells was accom-

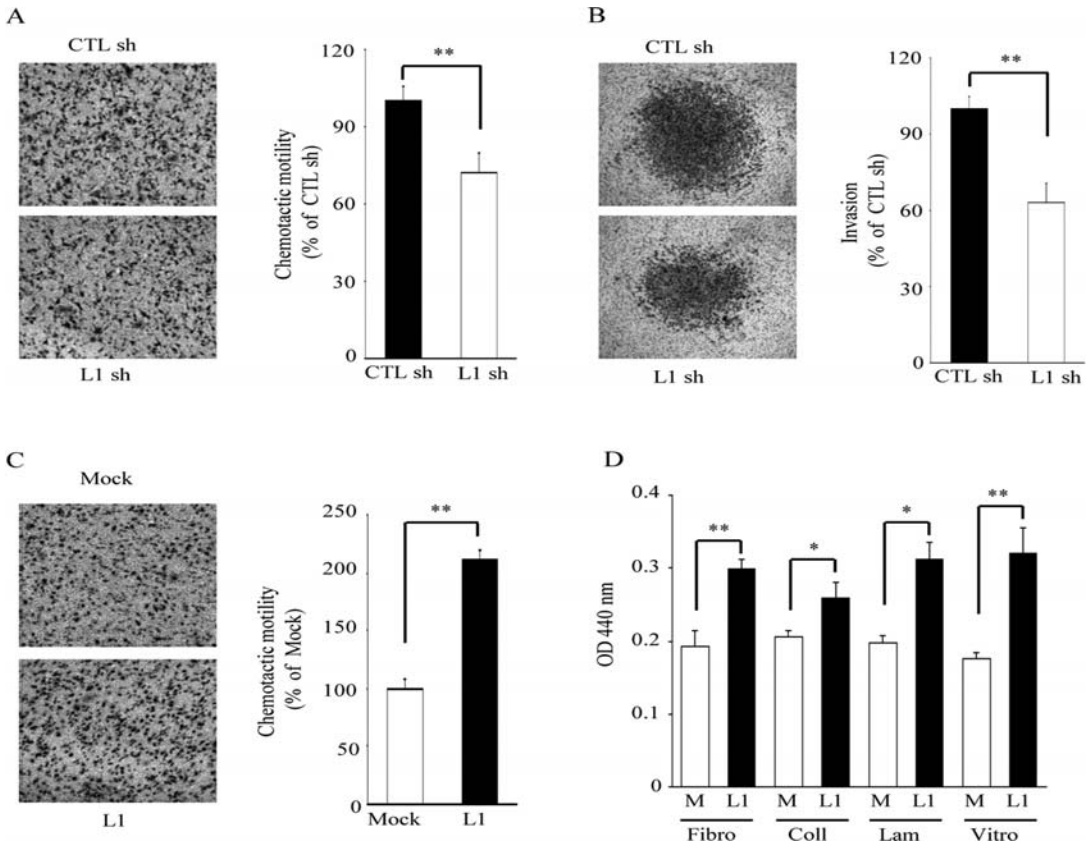

Figure 2. L1 promotes GBC cell migration, invasion and adhesiveness. (A-C) Migration (A and C) and invasion (B) were analyzed as described in Materials and methods. Three independent experiments were performed in duplicate. Data are expressed as the mean ± SD [**P<0.01 vs. control (CTL) sh (A and C) or vs. mock (B and D); *P<0.05 vs. mock]. (D) Effect of L1 overexpression on adhesion of SNU308 cells to extracellular matrix proteins. Attachment of mock-transfected or L1-overexpressing SNU308 cells to plastic plates coated with either fibronectin (Fibro), collagen (Coll), laminin (Lam) or vitronectin (Vitro) was determined using a WST1 assay (Materials and methods). M, mock. Data reflect the means of triplicates ± SD.

plished by lentivirus-mediated transduction and expression of an L1 mRNA-specific shRNA or full-length human L1 expression plasmid, respectively. The GBC cell lines stably expressing low or high levels of L1 were selected, and the relative expression level of L1 was confirmed by RT-PCR (data not shown) and Western blot analysis (Fig. 1A). As shown in Fig. 1, stable depletion of L1 caused a significant decrease in the growth rate and DNA synthesis of JCRB1033 cells (Fig. 1B and C), whereas the growth rate and DNA synthesis were higher for L1-overexpressing cells than for mock-transduced cells (Fig. 1D and E). Similar effects were observed under low-serum conditions, but not in the presence of 10% FBS (data not shown). To determine the effect of L1 on the colony-forming ability of GBC cells, we performed an *in vitro* soft agar assay. Down-regulation of L1 decreased anchorage-independent cell growth of GBC cells, whereas up-regulation of L1 increased it (Fig. 1F and G).

L1 promotes migration, invasion and adhesiveness of GBC cells. It has been shown that L1 is necessary for the motility and invasiveness of several types of tumors (9,12). We therefore examined the motility and invasiveness of GBC cell clones using transwell filter assays. As shown in Fig. 2A and B, GBC cells with stable L1 knockdown displayed significantly decreased motility and invasiveness, determined using matrigel-coated filters, compared with CTL sh cells. Consistently, overexpression of L1 in SNU-308 cells led to enhanced motility and adhesion to matrix components such as fibronectin, collagen, laminin and vitronectin (Fig. 2C and D), although we were unable to assess the effect of L1 knockdown on cell adhesiveness because the knockdown cells exhibited very rapid adhesion. These results indicate that L1 expression in GBC cells plays an important role in their motility, invasiveness and adhesiveness.

L1 regulates phosphorylation of AKT and FAK, but not ERK, in GBC cells. Recent studies have demonstrated that L1 plays a role in cancer by activating different signaling pathways that contribute to tumor progression (14). Indeed, ERK, AKT and FAK kinases are key signaling partners associated with transmitting signals that induce cell growth, migration, invasion and survival of tumor cells and are involved in signaling through L1 (15-17). We therefore examined the effect of L1 on phosphorylation of these signaling molecules in L1-knockdown or -overexpressing cells. As shown in Fig. 3A and B, the phosphorylation of FAK and AKT was down-regulated in the L1-knockdown cells but enhanced in L1-overexpressing cells. However, no significant reduction in phosphorylation of ERK was observed, implying that the impact of L1 on intracellular signaling may be tumor type-dependent.

L1 regulates diverse cellular functions via the PI3K/AKT and FAK signaling pathways, independent of ERK in GBC cells.

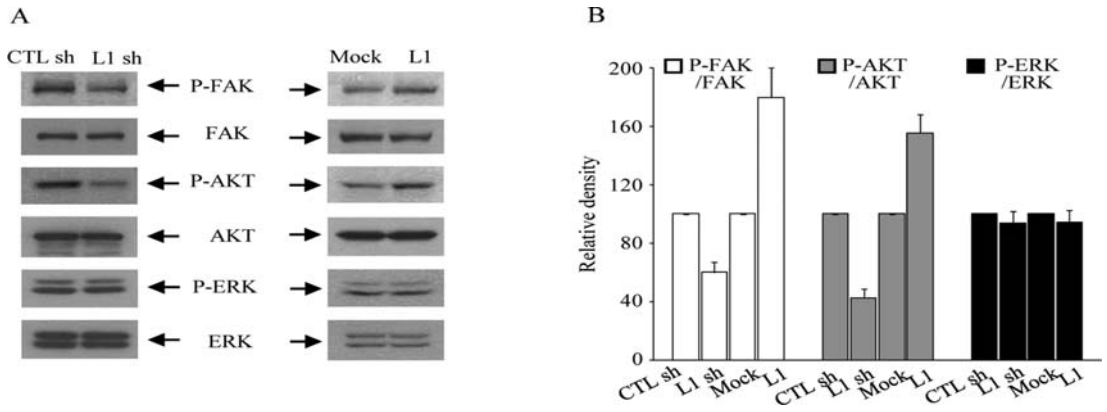

Figure 3. L1 activates intracellular signaling molecules in GBC cells. (A) Stable transfectants were seeded in medium containing 1% FBS. After 48 h, phosphorylation of FAK, AKT and ERK was analyzed by Western blotting. Data are representative of three independent experiments. (B) Western blots were quantified by densitometry, and data reflect the relative ratio of P-FAK:FAK, P-AKT:AKT and P-ERK:ERK.

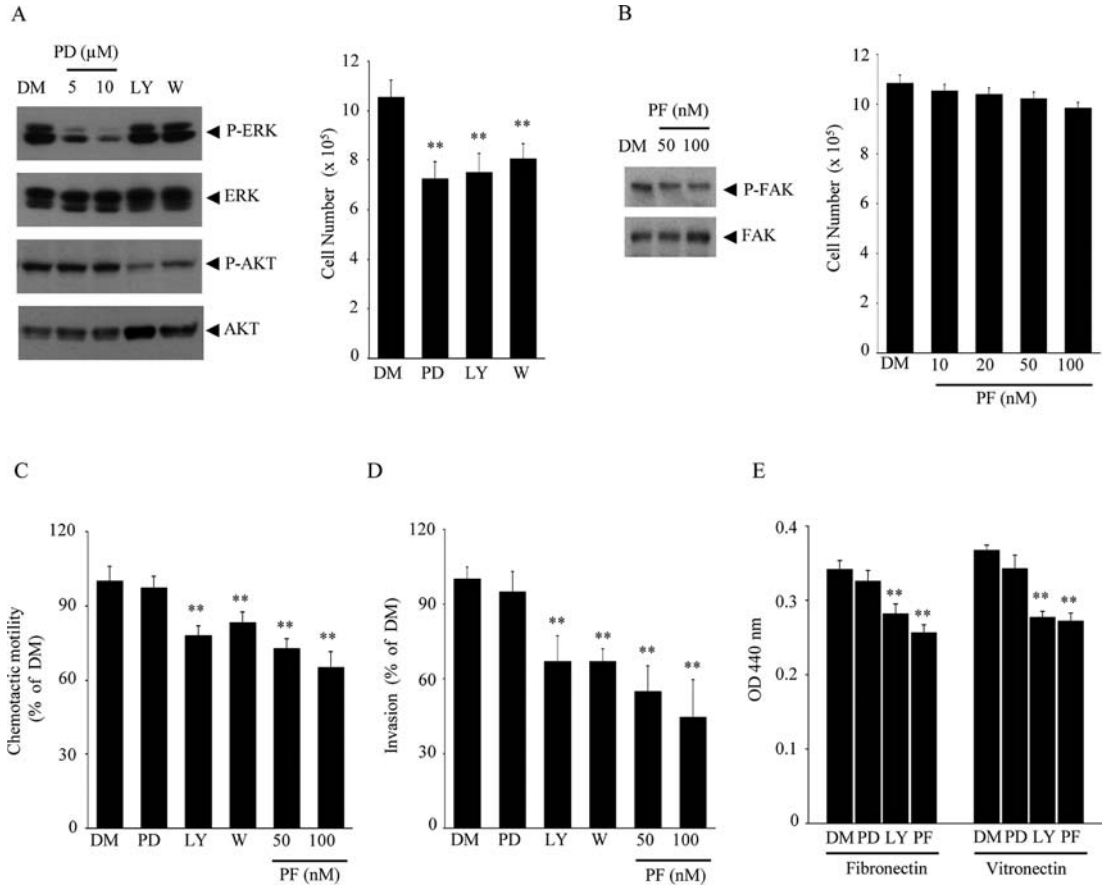

Figure 4. The involvement of L1-mediated FAK and PI3K/AKT signaling pathways in motility, invasiveness and adhesiveness of GBC cells. (A and B) JCRB1033 cells were seeded at a density of $2x10^5$ cells/well in 6-well dishes. After 24 h, the cells were incubated with PD98059 (10 μM), wortmannin (100 nM), LY294002 (5 μM) or PF573228 (10-100 nM) for 72 h and viable cells were counted in a cell counter. Three independent experiments were performed in duplicate. DM, DMSO; PD, PD98059; LY, LY294002; W, wortmannin; and PF, PF573228. Data are expressed as mean ± SD (**P<0.01 vs. DM). Western blot analysis showed that the inhibitors used in this experiments suppressed the signaling pathways (left panels of A and B). JCRB1033 cells (C and D) or SNU-308 cells (E) were incubated with PD98059 (10 μM), wortmannin (100 nM), LY294002 (5 μM) or PF573228 (50 or 100 nM) for 1 h. Migration (C), invasion (D) and adhesion (E) assays were performed as described in Materials and methods. Three independent experiments were performed in duplicate. Data are expressed as the mean ± SD (**P<0.01 vs. DM).

To verify whether L1-regulated signaling molecules are involved in GBC tumor progression, we tested the effects of inhibitors of ERK, FAK and PI3K, which are subsequently linked to the AKT pathway, on the growth, motility and invasiveness of GBC cells. The PI3K inhibitors LY294002 and wortmannin specifically reduced the growth, motility, and invasiveness of JCRB1033 cells (Fig. 4). Notably, the ERK inhibitor PD98059 specifically inhibited cell growth, whereas PF573228, a potent FAK inhibitor, had no significant effect on growth (Fig. 4A and B). In contrast, PF573228

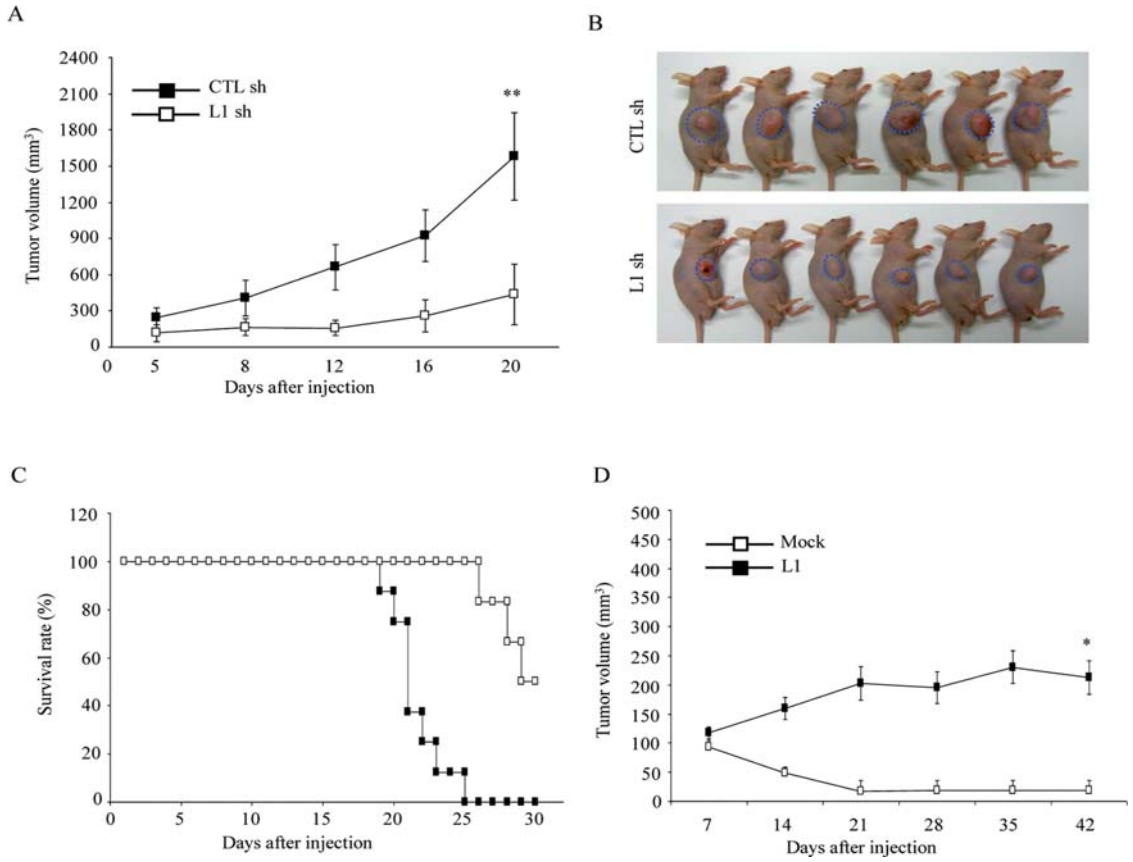

Figure 5. Suppression of L1 markedly reduces tumor growth whereas L1 overexpression confers tumorigenicity of GBC cell xenografts in nude mice. JCRB1033 cells transfected with control (CTL) sh or L1 sh (A through C) or SNU-308 cells transfected with mock or L1 were injected into the right flank of 7-week-old nude mice. (A and D) Tumor growth was monitored on the indicated days. Results represent mean tumor volume ± SD (n=8 animals). **P<0.01 vs. CTL sh group; *P<0.05 vs. mock group. (C) Analysis of survival rate. The percentage of surviving mice was determined by monitoring survival of mice with tumors >2000 mm^3 for 30 days.

markedly inhibited the motility and invasiveness of JCRB1033 cells (Fig. 4C and D). Consistently, treatment of SNU-308 L1-overexpressing cells with the PI3K inhibitors and the FAK inhibitor resulted in significant inhibition of L1-mediated cell proliferation and motility (data not shown). The PI3K and FAK inhibitors also significantly inhibited L1-mediated adhesion to fibronectin or vitronectin, but the ERK inhibitor did not (Fig. 4E). Taken together, these results indicate that in GBC cells, L1 regulates GBC cell growth, motility, and invasiveness via the PI3K/AKT and FAK signaling pathways, independent of ERK.

L1 potently promotes tumor growth in a GBC xenograft model. To evaluate the effect of L1 on tumor development *in vivo*, L1-knockdown or -overexpressing cells were injected into the right flank of nude mice and tumor formation was monitored. As shown in Fig. 5, a complete delay in tumor growth was observed in mice with L1 knockdown cell xenografts compared to those injected with GBC cells transfected with control shRNA. At 20 days post-injection, the average tumor volume was 1580.4±360 mm^3 in mice injected with control shRNA cells and 434.8±251 mm^3 in mice injected with L1 shRNA cells (Fig. 5A and B). Thus, tumor volume was decreased by 73% (P<0.05) in the presence of L1 shRNA. The increase in survival conferred by L1 knockdown was statistically significant when compared with the control group (P<0.05 vs. CTL sh group) (Fig. 5C). All mice inoculated with L1-overexpressing cells formed tumors, whereas mice injected with mock-transduced cells did not develop tumors (Fig. 5D). Taken together, these results suggest that L1 promotes the growth and tumorigenicity of GBCs.

Discussion

The expression of L1 in cancer was first discovered in malignant melanoma cells (28) and was subsequently found in various other cancer cells. L1 was detected in 79% of human ovarian cancer samples comprising all histological types (11,29). Similarly, L1 was detected in ~70% of colon cancer tumor samples, although other studies demonstrated that L1 expression was less common in various cancers (11-15). In addition, L1 was expressed in 46% of clear-cell renal cell carcinomas and in 28% of papillary renal cell carcinomas (9,30), suggesting that L1 plays an active role in cancer progression. In particular, numerous studies have shown that forced expression of L1 in normal and cancer cell lines increases cell motility and invasiveness *in vitro* and in animal models *in vivo*. Interestingly, L1 was also shown to be aberrantly expressed in malignant tumors of the biliary tract (13,31), implying that L1 is closely associated with develop-

ment and progression of biliary tract tumors. However, the exact role of L1 in GBC cells has not been previously elucidated.

In this study, we demonstrated that L1 is expressed in GBC cells and plays a crucial role in GBC progression. Two GBC cell lines, JCRB1033, expressing high levels of L1, and SNU-308, lacking L1 expression, in addition to GBC cell clones with stable knockdown (JCRB1033-derived) or overexpression (SNU-308-derived) of L1 were generated. Stable depletion of L1 caused a significant decrease in GBC cell proliferation, migration, invasion and intracellular signaling. In contrast, overexpression of L1 in GBC cells enhanced these cellular activities. Our data also demonstrate the prominent role of L1 in tumorigenicity of GBC cells *in vivo* using an animal model.

L1 has been shown to activate signaling pathways in several cell types, leading to alteration of various cellular functions. L1-mediated neuroprotection was associated with increased ERK and AKT activity and inhibition of caspases. L1 was also linked to activation of ERK and FAK, resulting in apoptotic protection in ovarian carcinomas (17). Indeed, we observed that L1 induced the phosphorylation of AKT and FAK and that L1-mediated activation of these factors was critical for cell growth, motility, invasiveness or adhesiveness of GBC cells. Unexpectedly, we did not find any increase or decrease of ERK phosphorylation in L1-overexpressing or knockdown GBC cells, implying that the impact of L1 on intracellular signaling may vary with different tumors. However, we clearly observed that the inhibition of ERK significantly inhibited GBC cell growth, indicating that although L1 is directly involved in the growth of GBC cells through an AKT-dependent signaling pathway, ERK signaling is also associated with the growth of GBC cells, independently from L1.

Because L1 expression is mostly observed in cancer tissues but rarely in normal tissues, and because it is localized to the cell surface, L1 may be a useful marker for diagnosis of patients with advanced stages of cancer and may represent a new target for therapeutic intervention. Recently, a monoclonal antibody against L1 was shown to significantly inhibit the tumor growth and migration of ovarian carcinomas in nude mice (32), and radioimmunotherapy with ^{67}Cu in conjunction with an L1 monoclonal antibody was effective for inhibiting growth of a metastatic ovarian carcinoma in a mouse model (33). Although, we did not evaluate the effect of an L1 monoclonal antibody in nude mice, our data clearly showed that suppression of L1 markedly reduced tumor growth and increased the survival of tumor-bearing mice in a GBC xenograft nude mouse model. Furthermore, L1 overexpression conferred tumorigenicity to GBC cells. These results strongly suggest the potential of L1 as a therapeutic target for GBC.

In conclusion, the present study provides the first evidence that L1 is expressed in GBCs and plays a crucial role in cell growth, motility, invasiveness and adhesiveness of GBC cells in addition to regulation of intracellular signaling molecules such as AKT and FAK. These results suggest that elevated L1 expression in GBCs may lead to GBC cell activation and disease progression. Therefore, L1 may serve as a potential therapeutic target for GBC treatment.

Acknowledgements

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