L1 Cell Adhesion Molecule is a Novel Therapeutic Target in Intrahepatic Cholangiocarcinoma

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Statement of translational relevance

Intrahepatic cholangiocarcinoma (ICC), a highly malignant hepatobiliary cancer, has a poor prognosis and is refractory to conventional therapies. Thus, new therapeutic strategies for ICC are urgently needed. In this study, we identified that L1 is expressed in ICC and plays an important role in the tumor progression of ICC by enhancing cell proliferation, migration, invasion, and survival. In addition, we observed that L1 shRNA or anti-L1 mAb reduced ICC tumor growth in nude mice. Therefore, suppression of L1 expression or inhibition of L1 may be a new therapeutic strategy for ICC therapy. Especially, anti-L1 mAb may be practically applied to the treatment of ICC.
Abstract

Purpose: Intrahepatic cholangiocarcinoma (ICC), a highly malignant hepatobiliary cancer, has a poor prognosis and is refractory to conventional therapies. The aim of this study is to discover a novel molecular target for the treatment of ICC.

Experimental Design: To discover novel cancer-associated membrane antigens expressed in ICC cells, we generated monoclonal antibodies (mAbs) by immunizing mice with intact ICC cell lines and screened for those that bind to the plasma membrane of ICC cells but not to normal cells. The mAb A10-A3 was selected and its target antigen was identified as the L1 cell adhesion molecule. Expression of L1 in ICC was evaluated by immunohistochemical analysis of tumor samples from 42 ICC patients. The functional significance of L1 expression in the tumor progression of ICC was investigated by L1 suppression, L1 overexpression, and antibody treatment.

Results: L1 was not expressed in normal hepatocytes and intrahepatic bile duct epithelium but highly expressed in 40.5% of ICC patients, remarkably at the invasive front of the tumors. Suppression of L1 with shRNA significantly decreased proliferation, migration, and invasion of ICC cells in vitro. Consistently, L1 overexpression in ICC cells enhanced proliferation, migration, invasion, and apoptosis resistance. In addition, L1 shRNA or anti-L1 mAb significantly reduced the tumor growth in nude mice bearing ICC xenograft.
Conclusions: We identified that L1 is expressed in ICC. L1 plays an important role in the tumor progression of ICC by enhancing cell proliferation, migration, invasion, and survival. L1 may represent a novel therapeutic target for ICC.
Introduction

Cholangiocarcinoma (CC) is a malignant tumor that arises from the bile duct epithelium. CC is classified into intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC). ICC occurs at a higher incidence in Southeast Asia than in Europe and North America, but the incidence and mortality rates are increasing worldwide (1-3). The prognosis of CC is poor due to the lack of early diagnosis and because the tumor is refractory to conventional chemotherapy and radiation treatment (4).

Surgical resection is the only way to cure the disease (5, 6). However, at the time of diagnosis, about 70% of CC patients have occult metastasis or advanced local disease which precludes curative resection. Thus, new therapeutic strategies for CC are urgently needed.

The L1 cell adhesion molecule is a 200-220 kDa transmembrane glycoprotein consisting of 6 immunoglobulin (Ig)-like domains followed by 5 fibronectin-type III repeats, a transmembrane domain, and a short cytoplasmic tail (7, 8). L1 was first 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 (9-12). L1 expression is found in other cell types such as lymphoid and myelomonocytic cells, kidney tubule epithelial cells, and intestinal crypt cells (13-17). Recent reports have shown that L1 is also expressed in several types of cancers including colon carcinoma, ovarian and uterine carcinomas, malignant gliomas, recurrent neuroblastoma, cutaneous...
malignant melanoma, and renal cell carcinoma, and that its expression correlates with tumor progression and metastasis (18-22). However, L1 expression in ICC has not been reported.

There have been reports that L1 promotes many cellular activities by homophilic binding of its extracellular domains and heterophilic binding to other cell adhesion molecules, extracellular matrix molecules, and cell surface receptors (23-26). Ectopic expression of L1 in carcinoma cells such as colon carcinoma, melanoma, or ovarian carcinoma enhances their migration, invasion, and tumorigenesis (22, 24, 27). The extracellular domain of L1 can be shed from the cell surface via proteolytic cleavage and stimulate the migration and survival of tumor cells through autocrine/paracrine binding to integrins (28-30). Recently, monoclonal antibodies (mAb) against L1 inhibited the growth of several L1-positive human tumor cell lines in vitro (31) and the tumor growth and dissemination of ovarian carcinomas in nude mice (32), suggesting L1 as a potential anticancer drug target. Moreover, a recent report showed that L1 is required for maintaining the growth and survival of CD133+ glioma cells and thus it may represent a cancer stem cell-specific therapeutic target for the treatment of malignant gliomas (33).

In this study, we identified that L1 is expressed in ICC by immunizing mice with human ICC cell lines and generating a mAb (A10-A3) with specificity for L1. Immunohistochemistry of tumor samples from 42 ICC patients using A10-A3 revealed that L1 was not expressed in normal hepatocytes or intrahepatic bile duct epithelium but highly expressed in 40.5% of the ICC cases. Suppression of endogenous L1 levels in ICC cells by L1 shRNA decreased cell proliferation, migration, and invasion as
well as intracellular signaling, while overexpression of L1 in the cells enhanced the cellular activities. In addition, L1 shRNA or anti-L1 mAb reduced the tumor growth in nude mice bearing ICC xenograft. The results suggest that L1 plays an important role in tumor progression of ICC and can serve as a novel therapeutic target for ICC.

Materials and Methods

Cell lines and cell culture. Choi-CK (adenomatous) and SCK (sarcomatoid) cell lines, established from Korean ICC patients (34), and ACHN human renal cell adenocarcinoma cells were grown in DMEM (Invitrogen) containing 10% FBS (Hyclone), and SKOV3 human ovary adenocarcinoma cells were grown in McCoy’s 5A medium (Invitrogen) with 10% FBS under cell culture conditions (5% CO₂, 95% relative humidity, 37°C). Human hepatocytes and HUVEC primary cultures were obtained from Cambrax, and human PBL were isolated from human blood by Ficoll density gradient.

Generation of mAbs. BALB/c mice were cared for according to the institutional guidelines of the Korea Research Institute of Bioscience and Biotechnology and were immunized through intraperitoneal injection with 2 x 10⁶ Choi-CK and SCK cells. Hybridomas were generated, as described previously (35). The culture supernatants of hybridomas were tested for reactivity to the ICC cells by flow cytometry, and each positive clone was isolated after two subcloning steps. Isotype of each mAb was determined using...
the mouse immunoglobulin isotyping ELISA kit (BD Pharmingen). Each mAb was purified from the
culture supernatants of hybridomas by Protein G-Sepharose column chromatography, as described
previously (35).

**A10-A3 target identification.** Cell surface biotinylation and immunoprecipitation of the antigen
recognized by A10-A3 or UJ127.11 (UJ127, Abcam) was performed, as described previously (35). The
immunoprecipitated protein was resolved on a SDS-PAGE gel, and the protein band corresponding to
approximately 200-kDa was excised and subjected to digestion and subsequent mass spectrometric
analyses, as described previously (35).

**Flow cytometry.** Cells were incubated with each mAb for 60 minutes at 4°C. After washing twice
with PBA, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse
immunoglobulin (Ig) (BD Pharmingen) for 30 minutes at 4°C. Propidium iodide (PI)-negative cells were
analyzed for antibody binding using FACSCalibur (BD Immunocytometry System) and Cell Quest
software (BD Immunocytometry System).

**Immunofluorescence staining.** Cells were cultured on gelatin-coated cover slips in DMEM
containing 10% FBS, fixed with 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. After
being washed in PBS, the slides were blocked with 10% horse serum in 1% PBA for 2 hours, and the cells
were incubated with A10-A3 (2 µg/ml) overnight followed by anti-mouse IgG-Cy3 (Jackson
ImmuoResearch) for 1 hour at 4°C, as described previously (35).
**Immunohistochemical analysis.** Surgically resected tumors from 42 ICC patients (28 males and 14 females) at the Chonnam National University hospital and Chungnam National University hospital were studied by immunohistochemistry using A10-A3. The mean age of the patients was 62 years (range: 41-76 years). All tumors were adenocarcinomas and defined as primary ICC arising from intrahepatic bile ducts. All specimens were fixed in formalin and embedded in paraffin. This study was approved by the Ethics Committee of the Chonnam National University hospital and Chungnam National University hospital, and written consent from all involved patients was obtained.

Immunohistochemistry was performed using the EnVision-HRP detection system (Dako). Mouse IgG1 isotype control excluding the primary antibody was used as a negative control, while peripheral nerve bundles present in sections served as an internal positive control. The immunohistochemical stains were evaluated by two independent pathologists JK and JL. The percentage of L1 positive tumor cells in ICC was assessed using a four-point scale (<5% = 0; ≥5% and <20% = +1; ≥20% and <50% = +2; ≥50% = +3) as described previously (36). The cases with 0 and +1 staining were considered as low expression group (LEG), while the cases with +2 and +3 staining were considered as high expression group (HEG). The correlation between the L1 expression and the clinicopathologic features (sex, age, histological grade) were analyzed by using the chi-square test or Fisher's exact test. $P < 0.05$ was considered statistically significant.
**Western blot analysis.** Cell lysates or immunoprecipitates from cells were subjected to SDS-PAGE and the protein bands in the gel were transferred to PVDF membranes (Chemicon). The membranes were incubated with primary antibodies followed by HRP-conjugated secondary antibodies (Santa Cruz), and the immunoreactive bands were visualized using a chemiluminescent substrate (GE Life Sciences).

**Short hairpin RNA L1 targeting and L1 overexpression in ICC cells.** Knock-down of L1 was achieved through the use of lentiviral vector-mediated short hairpin RNA (shRNA) interference using Mission RNAi system clones (Sigma-aldrich). For L1 overexpression, the cDNA encoding the L1 expressed in Choi-CK cells was cloned and its nucleotide sequence was determined. A lentiviral vector (Macrogen, South Korea) containing the full-length L1 cDNA and packaging vector were introduced into HEK293T cells using Lipofectamine, according to the manufacturer’s instructions, then the virus produced in the culture supernatants was added to Choi-CK or SCK cells along with 5 µg/ml polybrene. After 24 hours of incubation, the medium was removed and replaced with fresh medium containing 1.2 µg/ml puromycin. Puromycin-resistant clones were selected by incubating for 1 week in the medium containing puromycin. L1 expression level was analyzed by RT-PCR, Western blotting, and flow cytometry.

**Cell proliferation assay.** Cells (2 X 10^5) were grown in 6-well dishes in 2 ml medium containing 1% FBS for 72 hours, then viable cells were counted in a cell counter (Innovatis AG, Germany). Statistical significance of the data was evaluated using the Student's t test.
Migration and invasion assays. Migration and invasion of ICC 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 DMEM medium containing 2% FBS (SCK) or 5% FBS (Choi-CK) was placed in the lower wells. ICC cells were incubated for 24 hours in DMED containing 1% FBS and trypsinized and suspended at a final concentration of 1 x 10^6 cells/ml in DMED containing 1% FBS. One hundred µl of the cell suspension was loaded into each of the upper wells. The chamber was incubated at 37°C for 18 hours (migration) or 24 hours (invasion). Cells were fixed and stained with H&E. Nonmigrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and chemotaxis was quantified by counting with an optical microscope (x200) the cells that migrated to the lower side of the filter. Eight fields were counted for each assay.

Apoptosis assay. Choi-CK and SCK cells were treated with different concentrations (0 - 10 µg/ml) of gemcitabine (Yuhan, South Korea) in DMEM containing 10% FBS for 72 hours and viable cells were counted (Supplementary Fig. 4). For apoptosis assay, the cells (2 X 10^3) were seeded in 96-well plates and cultured overnight, then incubated with 0.5 µg/ml gemcitabine in the medium for 24 hours. The treated cells were lysed and subjected to assay for caspase-3 activity using chromogenic substrate, Ac-DEVD-pNA, used as the substrates for caspase-3-like proteases, as described previously (37).
Xenograft nude mice assay. Nude mice (6 weeks old) were obtained from the Charles River Laboratories (Boston, MA, USA). Mice were housed under specific pathogen free conditions and were used in accordance with the guidelines of the Animal Care Committee at the KRIBB. Control or L1 shRNA expressing cells (5 X 10⁶) were inoculated subcutaneously into the right flank of each mouse (n=8 per group). Tumor growth was monitored at 2-4 day intervals by measuring the length and width of the tumor with a caliper and calculating tumor volume on the basis of the following formula: volume=0.523Lw², where L is length and W is width. The percentage of surviving mice was determined by monitoring the tumor growth-related events (tumor size>2,000 mm³) for 50 days. Survival curve was plotted against time after injection (Kaplan–Meier survival function) and compared using a log-rank test analysis (StatView software; Abacus Concepts Inc., Berkeley, CA, USA). Differences in survival were considered statistically significant when P-values were <0.05.

To examine anti-tumor activity of A10-A3 in vivo, Choi-CK (3 X 10⁶) were injected subcutaneously into nude mice (n=8 per group), and A10-A3 antibody at a dose of 10 mg/kg was injected intravenously three times per week beginning on day 8. On day 20, mice were sacrificed and the tumor volumes were measured. To determine the toxicity to the animals, the body weight of the animals was measured.

Statistical analysis. Data are presented as 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

Generation of an anti-L1 mAb A10-A3. In an effort to generate mAbs against novel cancer-associated antigens expressed on the surface of CC cells, we immunized mice with Choi-CK and SCK cells and screened for the mAbs that bound to the surface of the tumor cells, but not to normal cells, by flow cytometry. The result showed that a mAb, A10-A3 (IgG1, κ) bound to the ICC cells, but it did not bind to normal hepatocytes, PBL, or HUVECs (Fig. 1A). A10-A3 also bound to other human tumor cell lines such as SK-OV3 ovarian carcinoma and A375 melanoma, while it did not bind to ACHN renal carcinoma cells (Fig. 1A). A10-A3 antigen was localized to the membrane of the ICC cells in immunofluorescence staining (Fig. 1B), while it was not detected in the ACHN cells.

To identify the cell surface antigen recognized by A10-A3, the surface proteins of Choi-CK cells were biotinylated and immunoprecipitated with A10-A3. As a result, an approximately 200-kDa protein was detected (Fig. 1C). Subsequently, the 200-kDa protein was excised from the protein gel, digested, and subjected to Q-TOF tandem mass spectrometric analyses. Analysis of the resulting peptide sequences revealed that the 200-kDa protein was the L1 cell adhesion molecule (Supplementary Fig. 1). Finally, the specificity of A10-A3 for L1 was confirmed by Western blot analysis using a known anti-L1 mAb, UJ127 (32), which showed that the UJ127 reacted with the protein immunoprecipitated by A10-A3 (Fig. 1C).
**L1 expression in ICC.** L1 expression in ICC was evaluated by immunohistochemical analysis of tumor specimens from 42 ICC patients, comprising 14 well differentiated, 14 moderately differentiated, and 14 poorly differentiated ICC. As shown in Fig. 2A, L1 expression was not detected in normal hepatocytes or intrahepatic bile duct epithelium, while it was detected in the arteriolar endothelial cells in normal liver, consistent with the previous report (15). L1 expression in tumor cells was detected in well (Supplementary Fig. 2A), moderately (Fig. 2B), and poorly differentiated (Fig. 2C) ICC. According to the assessment of L1 expression level using a four-point scale (0, +1, +2, +3), 25 (59.5%) out of 42 cases showed 0 (18 cases) or +1 (7 cases) staining, while 17 cases (40.5%) showed +2 (10 cases) or +3 (7 cases) staining (Table 1), indicating that L1 was highly expressed in 40.5% of ICC. Remarkably, strong L1 expression was observed at the invasive front of tumor cells (Fig. 2D), but not in the central, differentiated area of the tumor (Supplementary Fig. 2B), similar to that seen in colorectal carcinomas and ovarian carcinomas (22, 24). High level expression of L1 was preferentially observed in poorly differentiated tumors, while low level expression in well differentiated tumors (Table 1). However, there was no correlation between L1 expression and histological grade, age, or sex (Table 1). In contrast with ICC, A10-A3 did not react with the tumor tissues from 25 hepatocellular carcinoma patients in immunohistochemistry, suggesting that L1 may not be expressed in hepatocellular carcinoma (Supplementary Fig. 2C and 2D).
Reduction of cell proliferation, migration, and invasion by L1 shRNA transfection. To verify that
L1 plays a crucial role in tumor progression of ICC, endogenous L1 expression was suppressed by L1
shRNA transfection and cellular properties of the transfected cells were compared with those of control
shRNA-transfected cells (Fig. 3). Suppression of L1 expression in the L1 shRNA-transfected cells was
confirmed by RT-PCR (data not shown) and Western blot analysis, and the reduced L1 level at the cell
surface was also confirmed by flow cytometry (Fig. 3A). The L1 shRNA significantly reduced the
proliferation, migration, and invasion of the cells (Fig. 3B through 3D). Also, phosphorylation of FAK
and AKT, which contribute to proliferation, migration, invasion, and survival of tumor cells (38, 39), were
down-regulated in the L1 shRNA-transfected cells (Fig. 3E). However, no significant reduction in ERK
phosphorylation was observed in the cells (Fig. 3E).

Enhancement of cell proliferation, migration, and survival by L1 overexpression. To further
investigate the role of L1 in ICC tumor progression, the cDNA encoding the L1 was cloned from Choi-
CK cells and its nucleotide sequence was determined. A non-neural isoform (40) of human L1 lacking the
neuron-specific YEGHH and RSLE was identified (data not shown). Choi-CK and SCK cells were stably
transfected with the isoform of human L1, and the cell lines showing high level expression of L1 were
selected. The elevated L1 level was confirmed by flow cytometry and Western blot analysis (Fig. 4A and
Supplementary Fig. 3A). L1 overexpressing ICC cells showed enhanced cell proliferation, migration, and
invasion (Fig. 4B through 4D, and Supplementary Fig. 3B and 3C) compared with the control cells.
In addition, the role of L1 in apoptosis resistance was verified by comparing the effect of gemcitabine, currently used in chemotherapy for CC (41), on the mock and L1-overexpressing ICC cells. Choi-CK and SCK cells were 40% viable when treated with 0.5 µg/ml gemcitabine for 72 hours (Supplementary Fig. 4). For apoptosis assay, the cells were treated with the same concentration of the drug for 48 hours, and caspase-3 activity in the treated cells was measured. As a result, caspase-3 activity was significantly decreased in the L1-overexpressing cells compared with that in mock cells (Fig. 4E), suggesting that L1 contributes to survival of ICC cells. Also, phosphorylation FAK and AKT were enhanced in the L1-overexpressing cells (Fig. 4F).

**L1 suppression reduced the tumor growth in ICC xenograft model.** We evaluated the effect of L1 suppression on tumor growth in vivo. As shown in Figure 5A, a significant delay in tumor growth was observed in nude mice bearing ICC xenografts transfected with L1 shRNA compared with the control shRNA. At 35 days after injection, tumors with control shRNA reached an average volume of 1297.9 ± 317 mm³, while those with L1 shRNA had an average volume of 647.9 ± 92 mm³, indicating that 50% decrease (P < 0.05) in tumor volume was achieved. Also, L1 suppression significantly increased survival of tumor-bearing mice compared with the control group (Fig. 5B).

**Inhibition of ICC tumor growth in nude mice by antibody treatment.** To investigate if anti-L1 mAb can inhibit the ICC tumor growth in vivo, A10-A3 that binds to the surface of ICC cells (Fig. 1) or mouse IgG (mIgG) as a negative control was i.v. injected into nude mice bearing Choi-CK xenografts. The study
was stopped at day 20. A10-A3 inhibited the tumor growth in nude mice without affecting body weight (Fig. 5C and 5D).

Discussion

Conventional therapies are not effective in ICC and prognosis of ICC is very poor, calling for the discovery of new molecular targets and therapeutic strategies for the cancer. Recent reports have shown that L1 expression correlates with tumor progression and metastasis of several other carcinomas (18-22). However, L1 expression in ICC has not been previously reported. In this study, we identified that L1 is expressed in ICC (Fig. 1). Immunohistochemical analysis of ICC tumors revealed that L1 was not expressed in normal hepatocytes and intrahepatic bile duct epithelium but highly expressed in 40.5% of the ICC patients (Fig. 2, Table 1). Remarkably, strong L1 expression was detected at the invasive front of ICC, speculating that L1 may play an important role in invasion of ICC. Indeed, suppression of endogenous L1 level in ICC tumor cells by L1 shRNA significantly decreased cell proliferation, migration, and invasion as well as phosphorylation of FAK and AKT (Fig. 3), while overexpression of L1 enhanced cell proliferation, migration, apoptosis resistance, and the signaling (Fig. 4). In addition, L1 suppression in ICC cells delayed tumor growth in nude mice and increased survival of tumor-bearing mice, while anti-L1 mAb inhibited the tumor growth in nude mice (Fig. 5). The results suggest that L1
plays a crucial role in the progression of ICC. Recent studies showed that ectopic expression of L1 in
HEK293 cells activates ERK and FAK pathway and enhances cell motility, invasion, and apoptosis
resistance (27, 42) or that L1 promotes ERK- and PI3K-dependent cell proliferation, invasion, and
apoptosis resistance in ovarian carcinoma cells (43). However, in the present study no significant change
in ERK phosphorylation was detected by suppression or overexpression of L1. The results suggest that
the impact of L1 on intracellular signaling may vary with different tumor cells. The present study first
shows that L1 is expressed in ICC cells and plays a crucial role in tumor progression of ICC. The results
from L1 shRNA or anti-L1 mAb treatment suggest that L1 may serve as a therapeutic target for ICC.

Anti-L1 mAbs have been evaluated for treatment of cancers (44). L1-11A antibody or chCE7
antibody labeled with radioisotope showed therapeutic effects in ovarian carcinoma or neuroblastoma
xenograft model (19, 32, 45). However, inhibitory effects of the antibodies on intracellular signaling for
proliferation were not analyzed in the studies. In our study, we could not observe significant inhibitory
effects of A10-A3 on intracellular signaling or proliferation in vitro, while we clearly observed
that A10-A3 did inhibit migration of SCK cells in vitro (data not shown), suggesting that the
anti-tumor activity of A10-A3 in vivo may be mainly due to immune effector functions. The
exact mechanisms of anti-tumor activities of A10-A3 in vitro and in vivo need to be explored
further. Also, it is valuable to verify that combination treatment with this antibody and
chemotherapeutic agents for CC such as gemcitabine, platinum-based agents, and 5-fluorouracil
enhances therapeutic efficacy, compared to treatment with either antibody or the drug. In addition, since L1 was detected in peripheral nerve bundles and in the collecting tubules of the kidney (46), toxicology profile of A10-A3 in cross-reacting species should be assessed in preclinical studies.

In conclusion, L1 is aberrantly expressed in ICC and plays an important role in tumor progression of ICC by enhancing cell proliferation, migration, invasion, and survival. L1 may serve as a therapeutic target in ICC.

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Figure Legends

Fig. 1. Identification of L1 expression in ICC. A, flow cytometry using A10-A3 or UJ127. B, immunofluorescence staining using A10-A3. Antibody staining is in red, with nuclear DAPI staining in blue. C, immunoprecipitation of the extracts from biotinylated Choi-CK cells using A10-A3 or UJ127, followed by Western blot analysis of the immunoprecipitates using Streptavidin-HRP (left panel). Immunoprecipitation of Choi-CK cell extracts using A10-A3, followed by Western blot analysis of the immunoprecipitated protein with UJ127 and anti-mouse IgG-HRP (right panel).

Fig. 2. Immunohistochemical staining of ICC using A10-A3. A, normal intrahepatic tissue showing negative L1 staining. The arrow indicates bile duct epithelium and the arrowheads indicate hepatocytes. The white arrowhead indicates arteriolar endothelial cells. B, moderately differentiated ICC showing +2 staining. C, poorly differentiated ICC showing +3 staining. D, invasive front of ICC.

Fig. 3. Reduction of ICC cell proliferation, migration, and invasion by L1 shRNA. A, SCK cells were stably transfected with CTL shRNA or L1 shRNA using lentivirus as described in Materials and Methods. The expression of L1 was determined by flow cytometry (upper panel) and Western blot analysis (lower panel) using A10-A3. Uncolored populations indicate A10-A3 staining while colored populations indicate control staining in the panel. B-D, proliferation (B), migration (C), and invasion (D) assays were performed and the results are shown.
performed as described in Materials and Methods. Three independent experiments were performed in duplicate. CTL indicates control. Data are expressed as means ± SD; **P <0.01, with respect to CTL shRNA. E, the shRNA-transfected cells were grown in medium containing 1% FBS. After 48 hours, phosphorylation of FAK, AKT, and ERK were analyzed. Blots are representative of 3 independent experiments. Densitometric analyses are presented as the relative ratio of P-FAK:FAK, P-AKT:AKT, and P-ERK:ERK (right panel).

**Fig. 4.** Enhancement of ICC cell proliferation, migration, invasion, and apoptosis resistance by L1 overexpression. A, overexpression of L1 in SCK was analyzed by flow cytometry (upper panel) and Western blotting (lower panel) using A10-A3. Uncolored populations indicate A10-A3 staining while colored populations indicate control staining in each panel. B-E, proliferation (B), migration (C), invasion (D), and caspase-3 activity (E) assays were performed as described in Materials and Methods. Three independent experiments were performed in duplicate. Data are expressed as means ± SD; *P <0.01, with respect to Mock. F, the mock or L1-transfected cells were grown in medium containing 1% FBS. After 48 hours, phosphorylation of FAK, AKT, and ERK were analyzed. Blots are representative of 3 independent experiments.
Fig. 5. L1 shRNA or anti-L1 mAb reduced tumor growth in nude mice. SCK cells transfected with CTL shRNA or L1 shRNA (A, B), or Choi-CK cells (C, D) were injected into the right flank of 7-week-old nude mice. A and C, mean tumor volume ± SD for n=8 animals. *P <0.05 versus L1 shRNA group (A) or mIgG treated group (C). B, analysis of survival rate. The percentage of survived mice was determined by monitoring the tumor growth-related events (tumor size > 2000 mm$^3$) for 50 days. D, body weight of tumor bearing mice.
Figure 1

A

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L1

IgG
Figure 3
Figure 4

A) Flow cytometry analysis showing the FL-1 emission profile for Mock and L1 cells.

B) Bar graph indicating the total cell numbers for Mock and L1 cells.

C) Bar graph showing the migrated cell numbers per field for Mock and L1 cells.

D) Bar graph illustrating the invaded cell numbers per field for Mock and L1 cells.

E) Bar graph depicting the Caspase-3 activity (Abs/mg protein/hour) for Choi-CK and SCK.

F) Western blot analysis comparing P-FAK, FAK, P-AKT, AKT, P-ERK, and ERK levels between Mock and L1 cells with and without Gemcitabine (0.5 µg/ml) treatment.
Figure 5

A

Tumor volume (mm$^3$)

- SCK CTL shRNA
- SCK L1 shRNA

Days after injection

B

Survival rate (%)

- SCK CTL shRNA
- SCK L1 shRNA

Days after injection

C

Tumor volume (mm$^3$)

- mlgG (10 mg/kg)
- A10-A3 (10 mg/kg)

Days after injection

D

Body weight (g)

- mlgG (10 mg/kg)
- A10-A3 (10 mg/kg)

Days after injection
Table 1. The correlation between L1 expression and clinicopathologic features in patients with intrahepatic cholangiocarcinoma (42 cases)

<table>
<thead>
<tr>
<th>Category</th>
<th>L1</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LEG(^*)</td>
<td>HEG(^†)</td>
</tr>
<tr>
<td>Sex</td>
<td>n(^†) (%)</td>
<td>n(^†) (%)</td>
</tr>
<tr>
<td>Men</td>
<td>17 (60.7%)</td>
<td>11 (39.3%)</td>
</tr>
<tr>
<td>Women</td>
<td>8 (57.1%)</td>
<td>6 (42.9%)</td>
</tr>
<tr>
<td>Age (Y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>62 &gt;</td>
<td>12 (60.6%)</td>
<td>8 (40.0%)</td>
</tr>
<tr>
<td>62 ≤</td>
<td>13 (59.1%)</td>
<td>9 (40.9%)</td>
</tr>
<tr>
<td>Histologic grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>10 (71.4%)</td>
<td>4 (28.6%)</td>
</tr>
<tr>
<td>Moderately</td>
<td>8 (57.1%)</td>
<td>6 (42.9%)</td>
</tr>
<tr>
<td>Poorly</td>
<td>7 (50.0%)</td>
<td>7 (50.5%)</td>
</tr>
</tbody>
</table>

*Low expression group, †High expression group, ‡Number.
Clinical Cancer Research

L1 Cell Adhesion Molecule is a Novel Therapeutic Target in Intrahepatic Cholangiocarcinoma

Jeong-Ki Min, Jin-Man Kim, Shengjin Li, et al.

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