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 (mAb) 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 short hairpin RNA 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 short hairpin RNA 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.

Cholangiocarcinoma (CC) is a malignant tumor that arises from the bile duct epithelium. CC is classified into intrahepatic CC (ICC) and extrahepatic CC. 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, ~70% of CC patients have occult metastasis or advanced local disease that precludes curative resection. Thus, new therapeutic strategies for CC are urgently needed.

The L1 cell adhesion molecule is a 200- to 220-kDa transmembrane glycoprotein consisting of six immunoglobulin (Ig)–like domains followed by five 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 stimulates the migration and survival of tumor cells such as colon carcinoma, melanoma, or ovarian carcinoma in nude mice. Therefore, suppression of L1 expression or inhibition of L1 may be a new therapeutic strategy for ICC therapy. Especially, anti-L1 monoclonal antibody may be practically applied to the treatment of ICC.

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 short hairpin RNA or anti-L1 monoclonal antibody 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 monoclonal antibody may be practically applied to the treatment of 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% fetal bovine serum (FBS; Hyclone), and SK-OV3 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 human umbilical vascular endothelial cell (HUVEC) primary cultures were obtained from Cambrex, and human peripheral blood lymphocytes (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 immunized through i.p. injection with 2 × 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 Ig 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 done, as described previously (35). The immunoprecipitated protein was resolved on a SDS-PAGE gel, and the protein band corresponding to ~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 PBS, the cells were incubated with FITC-conjugated anti-mouse Ig (BD Pharmingen) for 30 minutes at 4°C. Propidium iodide–negative cells were analyzed for antibody binding using FACS Calibur (BD Immunocytometry System) and CellQuest software (BD Immunocytometry System).

Immunofluorescence staining

Cells were cultured on gelatin-coated coverslips 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% PBS for 2 hours, and the cells were
incubated with A10-A3 (2 μg/mL) overnight followed by anti-mouse IgG-Cy3 (Jackson ImmunoResearch) 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 done using the EnVision-HRP detection system (Dako). Mouse IgG1 isotype control excluding the primary antibody was used as a negative control, whereas peripheral nerve bundles present in sections served as an internal positive control. The immunohistochemical stains were evaluated by two independent pathologists (J.K. and J.L.). 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, whereas the cases with +2 and +3 staining were considered as high-expression group. The correlation between the L1 expression and the clinicopathologic features (sex, age, and histologic grade) was analyzed using the χ² 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 polyvinylidene difluoride membranes (Chemicon). The membranes were incubated with primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology), and the immunoreactive bands were visualized using a chemiluminescent substrate (GE Life Sciences).

**shRNA L1 targeting and L1 overexpression in ICC cells**

Knockdown of L1 was achieved through the use of lentiviral vector–mediated 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) containing the full-length L1 cDNA and packaging vector were introduced into HEK293T cells using Lipofectamine, according to the manufacturer’s instructions, and 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 reverse transcription-PCR, Western blotting, and flow cytometry.

**Cell proliferation assay**

Cells (2 × 10⁵) were grown in six-well dishes in 2-mL medium containing 1% FBS for 72 hours, and then viable cells were counted in a cell counter (Innovatis AG). Statistical significance of the data was evaluated using the Student’s t test.

**Migration and invasion assays**

Migration and invasion of ICC cells were done using Transwell (Corning Costar) 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 of reconstituted basement membrane substance for invasion assay (Matrigel; BD Biosciences). The fresh DMEM containing 2% FBS (SCK) or 5% FBS (Choi-CK) was placed in the lower wells. ICC cells were incubated for 24 hours in DMEM containing 1% FBS and trypsinized and suspended at a final concentration of 1 × 10⁶/mL in DMEM containing 1% FBS. Cell suspension (100 μL) 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. Non-migrating 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 (×200) 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) in DMEM containing 10% FBS for 72 hours, and viable cells were counted (Supplementary Fig. S4). For apoptosis assay, the cells (2 × 10⁵) were seeded in 96-well plates and cultured overnight, and 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 substrate for caspase-3–like proteases, as described previously (37).

**Xenograft nude mice assay**

Nude mice (6 weeks old) were obtained from Charles River Laboratories. Mice were housed under specific pathogen-free conditions and used in accordance with the guidelines of the Animal Care Committee at the Korea Research Institute of Bioscience and Biotechnology. Control shRNA- or L1 shRNA–expressing cells (5 × 10⁶) were inoculated s.c. into the right flank of each mouse (n = 8 per group). Tumor growth was monitored at 2- to 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.). Differences in survival were considered statistically significant when P values were <0.05.

To examine antitumor activity of A10-A3 in vivo, Choi-CK cells (3 × 10⁶) were injected s.c. into nude mice (n = 8 per group), and A10-A3 antibody at a dose of 10 mg/kg was injected i.v. 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 done using one-way ANOVA followed by Student's t test. A value of P < 0.05 was considered significant.

**Results**

**Generation of the 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 the mAb A10-A3 (IgG1, κ) bound to the ICC cells, but it did not bind to normal hepatocytes, PBLs, or HUVECs (Fig. 1A). A10-A3 also bound to other human tumor cell lines such as SK-OV3 ovarian carcinoma and A375 melanoma, whereas it did not bind to ACHN renal carcinoma cells (Fig. 1A). A10-A3 antigen was localized to the membrane of ICC cells in immunofluorescence staining (Fig. 1B), whereas it was not detected in 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. Consequently, an ~200-kDa protein was detected (Fig. 1C). Subsequently, the 200-kDa protein was excised from the protein gel, digested, and subjected to quadrupole time-of-flight tandem mass spectrometric analyses. Analysis of the resulting peptide sequences revealed that the 200-kDa protein was the L1 cell adhesion molecule (Supplementary Fig. S1). 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 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, whereas 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-differentiated (Supplementary Fig. S2A), moderately differentiated (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 of 42 (59.5%) cases showed 0 (18 cases) or +1 (7 cases) staining, whereas 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. S2B), similar to that seen in colorectal carcinomas and ovarian carcinomas (22, 24). High-level expression of L1 was preferentially observed in poorly differentiated tumors, whereas low-level expression was observed in well-differentiated tumors (Table 1). However, there was no correlation between L1 expression and histologic 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. S2C and D).

**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 reverse transcription-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-D). In addition,

**Table 1. Correlation between L1 expression and clinicopathologic features in patients with ICC (42 cases)**

<table>
<thead>
<tr>
<th>Category</th>
<th>L1</th>
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<tr>
<td></td>
<td>LEG, n (%)</td>
<td>HEG, n (%)</td>
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<tr>
<td>Sex</td>
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<td></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>
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<tr>
<td>Age (y)</td>
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<tr>
<td>&gt;62</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>
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<tr>
<td>Histologic grade</td>
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<tr>
<td>Well</td>
<td>10 (71.4%)</td>
<td>4 (28.6%)</td>
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<tr>
<td>Moderately</td>
<td>8 (57.1%)</td>
<td>6 (42.9%)</td>
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<tr>
<td>Poorly</td>
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Abbreviations: LEG, low-expression group; HEG, high-expression group.
phosphorylation of focal adhesion kinase (FAK) and AKT, which contribute to proliferation, migration, invasion, and survival of tumor cells (38, 39), was downregulated in the L1 shRNA–transfected cells (Fig. 3E). However, no significant reduction in extracellular signal-regulated kinase (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 nonneural 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; Supplementary Fig. S3A). L1-overexpressing ICC cells showed enhanced cell proliferation, migration, and invasion (Fig. 4B-D; Supplementary Fig. S3B and C) 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. S4). 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. Consequently, 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. In addition, phosphorylation FAK and AKT was 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 Fig. 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 1,297.9 ± 317 mm³, whereas those with L1 shRNA had an average volume of 647.9 ± 92 mm³, indicating that a 50% decrease ($P < 0.05$) in tumor volume was achieved. In addition, 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 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 D).

**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...
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). Immuno-histochemical 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, suggesting 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), whereas overexpression of L1 enhanced cell proliferation, migration, apoptosis resistance, and signaling (Fig. 4). In addition, L1 suppression in ICC cells delayed tumor growth in nude mice and increased survival of tumor-bearing mice, whereas 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 phosphatidylinositol 3-kinase–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 effect 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, whereas we clearly observed that A10-A3 did inhibit migration of SCK cells in vitro (data not shown), suggesting that the antitumor activity of A10-A3 in vivo may be mainly due to immune effector functions. The exact mechanisms of antitumor activities of A10-A3 in vitro and in vivo need to be explored further. In addition, 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 with treatment with either antibody or the drug. In addition, because 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Ministry of Science and Technology of Korea grants TNM0200512, TNM0200621, and C1007150-01-01; Korea Research Institute of Bioscience and Biotechnology grant KGM3100612; and Samsung Biomedical Research Institute.

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Received 11/19/2009; revised 05/12/2010; accepted 05/16/2010; published OnlineFirst 05/25/2010.

References

21. No potential conflicts of interest were disclosed.
22. Grant Support

Ministry of Science and Technology of Korea grants TNM0200512, TNM0200621, and C1007150-01-01; Korea Research Institute of Bioscience and Biotechnology grant KGM3100612; and Samsung Biomedical Research Institute.

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Received 11/19/2009; revised 05/12/2010; accepted 05/16/2010; published OnlineFirst 05/25/2010.
L1 Cell Adhesion Molecule Is a Novel Therapeutic Target in Intrahepatic Cholangiocarcinoma

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