L1 Cell Adhesion Molecule Is a Novel Independent Poor Prognostic Factor of Extrahepatic Cholangiocarcinoma

Shengjin Li,1 Young Suk Jo,2 Jae-Hyek Lee,5 Jeong-Ki Min,3 Eung Seuk Lee,3 Taewoo Park,3,4 Jin-Man Kim,1 and Hyo Jeong Hong3,4

Abstract Purpose: Cholangiocarcinomas (CC) are associated with poor survival, but diagnostic markers and therapeutic targets have not yet been elucidated. We previously found aberrant expression of L1 cell adhesion molecule in intrahepatic CC and a role for L1 in the progression of intrahepatic CC. Here, we analyzed L1 expression in extrahepatic CC (ECC) and evaluated its prognostic significance.

Experimental Design: We examined L1 expression in tumors from 75 ECC patients by immunohistochemistry. We analyzed the correlations between L1 expression and clinicopathologic factors as well as patient survival.

Results: L1 was not expressed in normal extrahepatic bile duct epithelium but was aberrantly expressed in 42.7% of ECC tumors. High expression of L1 was detected at the invasive front of tumors and was significantly associated with perineural invasion (P < 0.01). Univariate analysis indicated that various prognostic factors such as histologic grade 3, advanced pathologic T stage and clinical stage, perineural invasion, nodal metastasis, and high expression of L1 were risk factors predicting patient survival. Multivariate analyses done by Cox’s proportional hazards model showed that high expression of L1 (hazard ratio, 2.171; 95% confidence interval, 1.162-4.055; P = 0.015) and nodal metastasis (hazard ratio, 2.088; 95% confidence interval, 1.159-3.764; P = 0.014) were independent risk factors for patient death.

Conclusions: L1 was highly expressed in 42.7% of ECC and its expression was significantly associated with perineural invasion. High expression of L1 and nodal metastasis were independent poor prognostic factors predicting overall survival in patients with ECC. (Clin Cancer Res 2009;15(23):OF1–7)

Cholangiocarcinomas (CC) are malignant tumors originating from the bile duct epithelium (1) and can be classified anatomically into intrahepatic CC (ICC) and extrahepatic CC (ECC; refs. 2–4). Complete surgical resection is currently the only way to treat and cure CCs. However, due to lack of early diagnosis, most patients with CC have regional or distant metastasis at clinical presentation (5). Furthermore, the overall 5-year survival rate, which includes patients having undergone surgical treatment, is less than 5%. This poor survival outcome might be due to the poor response of CC to conventional chemotherapy and radiation treatment. However, no specific diagnostic and therapeutic tools are currently available for CC because of limited insight into the molecular pathogenesis of CC. Therefore, the identification of molecular markers to determine patient survival and new effective therapeutic targets for CC is urgently needed.

L1 is a 200- to 220-kDa transmembrane glycoprotein (6, 7). L1 was first identified as a neural cell adhesion molecule that

Authors' Affiliations: 1Department of Pathology and Infection Signaling Network Research Center, Chungnam National University School of Medicine, 2Laboratory of Endocrine Cell Biology, Division of Endocrinology, Department of Internal Medicine, Chungnam National University Hospital, 3The Therapeutic Antibody Research Center, and 4Korea University of Science and Technology, Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea; and 5Department of Pathology, Chonnam National University Medical School, Gwangju, Korea. Received 4/27/09; revised 9/8/09; accepted 9/15/09; published OnlineFirst 11/17/09.

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Current address for S. Li: Oral Cancer Research Institute, Yonsei University College of Dentistry, Yonsei University, Seoul 120-749, Korea.

Requests for reprints: Hyo Jeong Hong, The Therapeutic Antibody Research Center, Korea Research Institute of Bioscience and Biotechnology, 52 Eoeun-dong, Yuseong-gu, Daejeon 305-333, Korea. Phone: 82-42-860-4122; Fax: 82-42-860-4597; E-mail: hjhong@kriibb.re.kr or Jin-Man Kim, Department of Pathology and Infection Signaling Network Research Center, Chungnam National University School of Medicine, Daejeon, Korea. Phone: 82-42-580-8237; Fax: 82-42-581-5233; E-mail: jinmank@knu.ac.kr.

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Translational Relevance

Extrahepatic cholangiocarcinoma (ECC) is a highly malignant hepatobiliary cancer with a poor prognosis. Here, we found that a significant proportion of tumors from ECC patients showed aberrant L1 expression. Furthermore, tumors with high L1 expression are more frequently accompanied with perineural invasion, and patients with high L1 expression had shorter survival duration than patients with low expression (15.3 ± 2.7 versus 35.3 ± 3.9; P < 0.001). Analysis of duration of stable disease showed that patients with high L1 expression showed poor prognosis (11.9 ± 2.4 versus 27.1 ± 3.6; P = 0.001). Univariate analyses revealed histologic grade 3, pathologic T stage III and IV, clinical stage III and IV, perineural invasion, nodal metastasis, and high L1 expression as significant prognostic factors. Multivariate analyses clearly showed that high L1 expression is an independent risk factor for patient death (hazard ratio, 2.171; 95% confidence interval, 1.162-4.055; P = 0.015). The data suggest that high L1 expression is related to tumor progression and may be a useful marker for predicting survival of ECC patients.

Materials and Methods

Western blot analysis for A10-A3 specificity. ECC cell lines SNU-245 (29) and SNU-1196 (29) were grown in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (Hyclone), and JCRB1032 cells showed L1 expression in several types of cancers such as malignant gliomas, recurrent neuroblastoma, malignant melanoma, ovarian and uterine carcinomas, renal cell carcinoma, colorectal cancer, and pancreatic adenocarcinoma and neuroendocrine carcinoma. Furthermore, its expression correlates with tumor progression and metastasis (16–26). In particular, L1 expression is a reported marker for poor prognosis and short survival in patients with ovarian carcinoma and colorectal cancer (24, 27). L1 may also play a role in the development of human colon cancer, as one study suggested that it may be a target of β-catennín–T-cell factor signaling, which contributes to tumor development during various stages of colon cancer (22). However, the expression and clinical effect of L1 in CC has not been previously investigated.

We previously generated a murine monoclonal antibody (A10-A3) against L1 and used this antibody to show that L1 is highly expressed in 63.6% of pulmonary large-cell neuroendocrine carcinomas (28). In the present study, we used A10-A3 in immunohistochemical analysis of tumor specimens from 75 patients with ECC and found that L1 is highly expressed in 42.7% of ECC tumors, especially in the invasive front. In addition, analyses of the correlation between L1 expression and prognostic factors as well as patient survival clearly showed that high expression of L1 was closely related to perineural invasion. Our findings suggest that L1 could function as a useful marker to predict patient survival.

Fig. 1. L1 expression in ECC. A, normal extrahepatic bile duct epithelium. B, well-differentiated ECC (example of +1 staining). C, moderately differentiated ECC (example of +2 staining). D, poorly differentiated ECC (example of +3 staining).
Patients and tissue specimens. We retrospectively investigated 75 patients (56 men and 19 women) with ECC who underwent primary surgical resection between May 1999 and October 2005 at the Chonnam National University hospital and Chumung National University hospital. The mean age of the patients was 65 y (range, 48-84). Tumor specimens were fixed in 10% formalin and embedded in paraffin; all tumors were adenocarcinoma and defined as primary ECC arising from extrahepatic bile ducts. All protocols were approved by the institutional review board.

Immunohistochemistry. Immunohistochemistry for L1 expression was done using purified A10-A3 and the EnVision-HRP detection system (DakoCytomation). Processing was mostly done at room temperature, except for incubation with A10-A3 at 4°C. Briefly, sections (4 μm thick) were cut from tumor tissue blocks mounted on slides and dried for 1 to 2 h at 56°C. The sections were deparaffinized in xylene and rehydrated in graded alcohol. After antigen retrieval in a pressure cooker with target retrieval solution (DakoCytomation) at full power for 4 min, tissue sections were treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase. Sections were incubated overnight with A10-A3 diluted with background reducing diluent (80 ng/mL; DakoCytomation) in a humid chamber at 4°C. Slides were then incubated with EnVision reagent for 30 min followed by 3,3′-diaminobenzidine chromogen for 5 min, counterstained with Mayer’s hematoxylin, and mounted. Mouse IgG1 control excluding the primary antibody was used as a negative control, whereas peripheral nerve bundles in the same sections served as an internal positive control. The entire stained sections were scanned at >200 magnification using a light microscope (Nikon E600).

Evaluation of immunohistochemical staining. The immunohistochemical results were evaluated by two independent pathologists (I-M.K. and J-H.L.). Specimens were considered immunopositive for L1 staining when the tumor cells showed clear evidence of membranous staining. The percentage of positively stained tumor cells was graded using a four-point scale (<5% = 0; ≥5% to <20% = +1; ≥20% to <50% = +2; ≥50% = +3) as described previously (28, 31). The cases with 0 and +1 staining scores were considered part of the low expression group (LEG), whereas those with +2 and +3 staining scores were considered the high expression group (HEG).

Histologic grading. The ECC tissue specimens were subjected to routine H&E staining. The WHO grading system was used to categorize the specimens into well-differentiated (G1), moderately differentiated (G2), and poorly differentiated (G3) adenocarcinoma. Thirteen cases were well differentiated, 43 cases were moderately differentiated, and 19 cases were poorly differentiated.

Statistical analysis. Group comparisons of categorical variables were done using the χ² test or linear by linear association. Comparisons of average means were evaluated with the independent samples t test. Differences were considered statistically significant if P < 0.05.

Results

Immunohistochemical analysis for L1 expression in ECC. We previously generated a murine monoclonal antibody A10-A3 against L1 (28). The specificity of A10-A3 against human L1 is shown by Western blot analysis using three ECC cell lines and human umbilical vascular endothelial cells as a negative control (Supplementary Fig. S1). In the present study, L1 expression in ECC was evaluated by immunohistochemical analysis of tissue specimens from 75 ECC patients using A10-A3. L1 was not expressed in normal epithelium of the extrahepatic bile duct (Fig. 1A), whereas its expression was detected in ECC tumor cells. Next, we assessed L1 expression level by determining the percentage of positively stained tumor cells using a four-point scale (0, +1, +2, +3). A total of 43 cases (57.3%) showed 0 (31 cases) or +1 (12 cases) staining (LEG), whereas 32 cases (42.7%) showed +2 (16 cases) or +3 (16 cases) staining (HEG), indicating that L1 is highly expressed in 42.7% of ECC tumors (Table 1). Representative cases of L1 expression in well-differentiated (Fig. 1B), moderately differentiated (Fig. 1C), and poorly differentiated (Fig. 1D) patients are shown in Supplementary Fig. S1.
and tumor progression, we classified variables such as histologic grade, pathologic T stage, and clinical stage into two categories; raw data are presented in Supplementary Table S1. Overall survival and tumor progression of 75 ECC patients were also analyzed. The median survival time of ECC patients was 16 months (range, 1-94 months), and the overall 5-year survival rate was 21.3% (16 of 75 cases), whereas the rate of stable disease at 5 years was 13.3% (10 of 75 cases). The survival curves according to L1 expression are shown in Fig. 3. The overall survival rates of the HEG and LEG were 3.1% and 34.9%, respectively (Fig. 3A; \( P < 0.001 \)), and the rates of stable disease for HEG and LEG were 0% and 23.3%, respectively (Fig. 3B; \( P < 0.01 \)). These analyses using the Kaplan-Meier method clearly showed the significant effect of L1 expression on clinical outcome.

To estimate the clinical significance of various prognostic factors that might influence survival and tumor progression in ECC, univariate analyses were done. As summarized in Table 2, histologic grade 3 (\( P = 0.001 \)), advanced pathologic T stage (\( P = 0.003 \) and clinical stage (\( P = 0.007 \)), positive perineural invasion (\( P = 0.009 \)) and lymph node metastasis (\( P = 0.005 \)), and high expression of L1 (\( P < 0.001 \)) were statistically significant risk factors affecting overall survival of ECC patients. About tumor progression, histologic grade 3 (\( P = 0.004 \)), advanced pathologic T stage (\( P = 0.02 \)), positive perineural invasion (\( P = 0.02 \)), lymph node metastasis (\( P = 0.02 \)), and high expression of L1 (\( P < 0.001 \)) were significant risk factors. To determine independent prognostic effects of these various factors, multivariate analyses were done using the Cox's proportional hazards model. Results showed that positive lymph node metastasis [hazard ratio (HR), 2.088; 95% confidence interval
(95% CI), 1.1587-3.764; \( P = 0.014 \) and high expression of L1 (HR, 2.171; 95% CI, 1.162-4.055; \( P = 0.015 \)) were independent risk factors predicting the overall survival of ECC patients (Table 3). Taken together, our findings indicate that L1 expression could be a useful marker to predict ECC patient survival.

**Discussion**

Recent reports have correlated high expression of L1 with progression and metastasis of several types of cancers, such as malignant gliomas, recurrent neuroblastoma, malignant melanoma, ovarian and uterine carcinomas, renal cell carcinoma, colorectal cancer, and pancreatic adenocarcinoma and neuroendocrine carcinoma (16–27). However, L1 expression in ECC and its correlation with tumor progression has not been reported. This study first shows that L1 is not expressed in normal extrahepatic bile duct epithelium but is highly expressed in a significant proportion (42.7%) of ECC (Fig. 1; Table 1). Remarkably, high expression of L1 was detected at the invasive front of the tumors (Fig. 2B), similar to colorectal carcinomas (22). In colorectal cancer, L1 was shown to be a target gene of \( \beta \)-catenin–T-cell factor signaling (22). Aberrant expression and nuclear accumulation of \( \beta \)-catenin could induce transcription of growth-promoting target genes such as cyclin D1 and c-myc (34, 35), and in later stages of tumorigenesis, \( \beta \)-catenin can contribute invasive and metastatic capacities to cancer progression by inappropriate induction of metalloproteinase and cell adhesion receptors (36, 37). Because aberrant expression and nuclear accumulation of \( \beta \)-catenin was previously observed in CC (38, 39), we speculate that L1, a novel target of \( \beta \)-catenin, might play a role in the tumorigenesis of CC.

Interestingly, we found that high L1 expression was significantly associated with positive perineural invasion (Fig. 2D), whereas expression was not related to histologic grade, tumor stage, venous/lymphatic invasion, lymph node metastasis, and distant metastasis (Table 1). Perineural invasion was shown to be a potential prognostic indicator in ECC in our study. First, perineural invasion was frequently observed in ECC, with 55 (73.3%) of 75 ECC cases showing positive perineural invasion. Second, positive perineural invasion was closely linked to high histologic grade and advanced pathologic T stage of ECC (data not shown). Taken together, high L1 expression could function as a useful indicator of tumor aggressiveness.

Univariate analyses indicated many risk factors for patient survival and tumor progression, such as high histologic grade, advanced pathologic T stage and clinical stage, positive perineural invasion and nodal metastasis, and high L1 expression (Table 2). Furthermore, multivariate analyses clearly showed that high L1 expression is an independent poor prognostic factor for overall survival as likely as lymph node metastasis.

**Table 2.** Univariate analyses of the associations of prognosis with various clinicopathologic parameters and L1 expression in patients with ECC

<table>
<thead>
<tr>
<th>Category</th>
<th>Overall survival</th>
<th>Tumor progression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>( P )</td>
</tr>
<tr>
<td>Women</td>
<td>0.921 (0.505-1.678)</td>
<td>0.790</td>
</tr>
<tr>
<td>Old age (≥65 y)</td>
<td>1.477 (0.880-2.477)</td>
<td>0.140</td>
</tr>
<tr>
<td>Histologic grade (G3)</td>
<td>2.651 (1.485-4.734)</td>
<td>0.001</td>
</tr>
<tr>
<td>Pathologic T stage (T3 + T4)</td>
<td>2.249 (1.306-3.873)</td>
<td>0.003</td>
</tr>
<tr>
<td>Clinical stage (III + IV)</td>
<td>2.390 (1.264-4.519)</td>
<td>0.007</td>
</tr>
<tr>
<td>Venous/lymphatic invasion</td>
<td>1.326 (0.791-2.222)</td>
<td>0.285</td>
</tr>
<tr>
<td>Perineural invasion</td>
<td>2.459 (1.257-4.811)</td>
<td>0.009</td>
</tr>
<tr>
<td>Nodal metastasis</td>
<td>2.158 (1.269-3.669)</td>
<td>0.005</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>1.137 (0.410-4.231)</td>
<td>0.640</td>
</tr>
<tr>
<td>High expression of L1</td>
<td>2.632 (1.571-4.476)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table 3. Multivariate analyses of the associations of prognosis with various clinicopathologic parameters and L1 expression in patients with ECC

<table>
<thead>
<tr>
<th>Category</th>
<th>Overall survival</th>
<th>Tumor progression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Women</td>
<td>1.051 (0.533-2.073)</td>
<td>0.886</td>
</tr>
<tr>
<td>Old age (≥65 y)</td>
<td>1.627 (0.911-2.908)</td>
<td>0.100</td>
</tr>
<tr>
<td>Histologic grade (G3)</td>
<td>1.638 (0.790-3.396)</td>
<td>0.185</td>
</tr>
<tr>
<td>Pathologic T stage (T3 + T4)</td>
<td>1.873 (0.995-3.528)</td>
<td>0.052</td>
</tr>
<tr>
<td>Clinical stage (III + IV)</td>
<td>1.194 (0.524-2.171)</td>
<td>0.673</td>
</tr>
<tr>
<td>Venous/lymphatic invasion</td>
<td>1.140 (0.66-1.97)</td>
<td>0.638</td>
</tr>
<tr>
<td>Perineural invasion</td>
<td>1.562 (0.708-3.443)</td>
<td>0.270</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td>0.729 (0.177-3.443)</td>
<td>0.662</td>
</tr>
<tr>
<td>Nodal metastasis</td>
<td>2.088 (1.159-3.764)</td>
<td>0.014</td>
</tr>
<tr>
<td>High expression of L1</td>
<td>2.171 (1.16-4.055)</td>
<td>0.015</td>
</tr>
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</table>

(Table 3; ref. 40). These data suggest that high L1 expression could be a useful prognostic marker to predict ECC patient survival. About the function of L1 in cancers, L1 can activate extracellular signal-regulated kinase and induce extracellular signal-regulated kinase–regulated genes that may contribute to self-sufficiency in growth, apoptosis evasion, limitless replicative potential, angiogenesis, insensitivity to antigrowth signal, and tissue invasion and metastasis (41). In addition, the extracellular domain of L1 can bind various extracellular matrix proteins and receptors, implying that L1 may also mediate cell–extracellular matrix interactions to promote cancer invasion and metastasis (42). Moreover, 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 (43,44). Recently, a monoclonal antibody against L1 was shown to significantly inhibit the tumor growth and migration of ovarian carcinomas in nude mice (45), suggesting that anti-L1 antibody may have the potential to be an anticancer agent in ECC therapy. Taken together, L1 might play a crucial role in progression of ECC and function as not only a useful prognostic marker but also a therapeutic target for the treatment of ECC.

In conclusion, we first report L1 expression in ECC and its correlation with tumor progression. High L1 expression was detected at the invasive front of ECC tumors and was significantly associated with perineural invasion. Our results show that L1 expression was an independent poor prognostic factor for survival in ECC patients, indicating that L1 can serve as a novel poor prognostic factor.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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