Expression of L1 Cell Adhesion Molecule Is a Significant Prognostic Factor in pT3-stage Gastric Cancer

YASUHIRO KODERA[1], HAYAO NAKANISHI[2], SEIJI ITO[3], KAZUNARI MISAWA[3],
YUICHI ITO[1], GORO NAKAYAMA[1], MASAHIKO KOIKE[1],
MICHITAKA FUJIWARA[1], YOSHITAKA YAMAMURA[3] and AKIMASA NAKAO[1]

[1]Department of Surgery II, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya, Aichi;
[2]Laboratory of Pathology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya, Aichi;
[3]Department of Gastroenterological Surgery, Aichi Cancer Center Hospital, Chikusa-ku, Nagoya, Aichi, Japan

Abstract. *Background: L1, a 200-220 kDa transmembrane glycoprotein of the immunoglobulin superfamily, has been shown to affect prognosis of various types of cancer and was shown to enhance peritoneal metastasis in ovarian cancer in vivo. Patients and Methods: Immunostaining with anti-L1 antibody was performed with 72 surgically resected pT3-stage gastric cancer specimens. Correlation of immunoreactivity with clinicopathological variables was evaluated and survival curves were compared between L1-positive and negative cases. Results: L1 was detected in 15 specimens (21%), more often among the intestinal-type cancer. No correlation was observed between L1 expression and presence of free cancer cells in the peritoneal cavity or development of peritoneal carcinomatosis during the follow-up. Nevertheless, prognosis of patients with L1-positive cancer was significantly inferior (p=0.024), particularly among the diffuse-type cancer cases. Conclusion: L1 was associated with poor outcome in gastric carcinoma. However, its association with the formation of peritoneal metastases or the presence of free cancer cells in the abdominal cavity was not observed. Further study to identify the role of L1 in gastric cancer progression and metastasis is warranted.*

Gastric cancer is a major health concern worldwide (1). Although prognosis of early-stage cancer has improved (2), outcome of advanced/metastatic disease remains dismal. Peritoneal carcinomatosis is the commonest pattern of disease failure in the Far East (3, 4). However, owing to the difficulty in early detection and absence of measurable lesions, gastric

cancer patients with peritoneal metastasis have not been adequate candidates for clinical trials testing new treatment modalities. The authors have explored peritoneal metastasis from the viewpoint of free cancer cells shed into the abdominal cavity, which has been detected in >65% of patients with serosa-positive gastric cancer (5). Some gastric cancer cases do not develop peritoneal metastases despite invasion of the serosal surface, and may be biologically different from those that generate peritoneal disease. Attempts to elucidate characteristics of cancer that metastasizes to the peritoneal lining are thus warranted, both to understand the mechanism behind metastasis and to seek effective treatment.

L1 cell adhesion molecule (L1) is a 200-220 kDa transmembrane glycoprotein of the immunoglobulin super family initially identified in neural cells (6). L1 was recently detected in ovarian and uterine carcinomas in a stage-dependent manner and its expression was found to be a valuable marker for poor prognosis (7). It is also correlated with tumor progression and metastasis of several other types of cancer, including malignant glioma (8), renal cell carcinoma (9), pancreatic neuroendocrine tumor (10), melanoma (11) and colorectal cancer (12, 13). Interestingly, it was observed exclusively in the invasion front of colorectal cancer, and was found abundantly in the culture medium of colon cancer cell lines when the cells were sparse whereas the expression diminished when the cells were dense (14), suggesting its role in cell proliferation and metastasis. Moreover, antibodies to L1 were shown to have therapeutic potential and reduce cell proliferation of human ovarian cancer cells *in vitro* and in a xenograft mouse model of peritoneal metastasis (15). These reports prompted the authors to ask whether the expression of L1 was also associated with the outcome of gastric cancer, and particularly with development of peritoneal carcinomatosis. To address this issue, the authors used a panel of surgically resected specimens consisting exclusively of serosa-positive (pT3-stage) cancer, and evaluated the expression of L1 through immunostaining.

Correspondence to: Yasuhiro Kodera, Department of Surgery II, Nagoya University Graduate School of Medicine, 65, Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan. Tel: +81 527442250, Fax: +81 527442255, e-mail: ykodera@med.nagoya-u.ac.jp

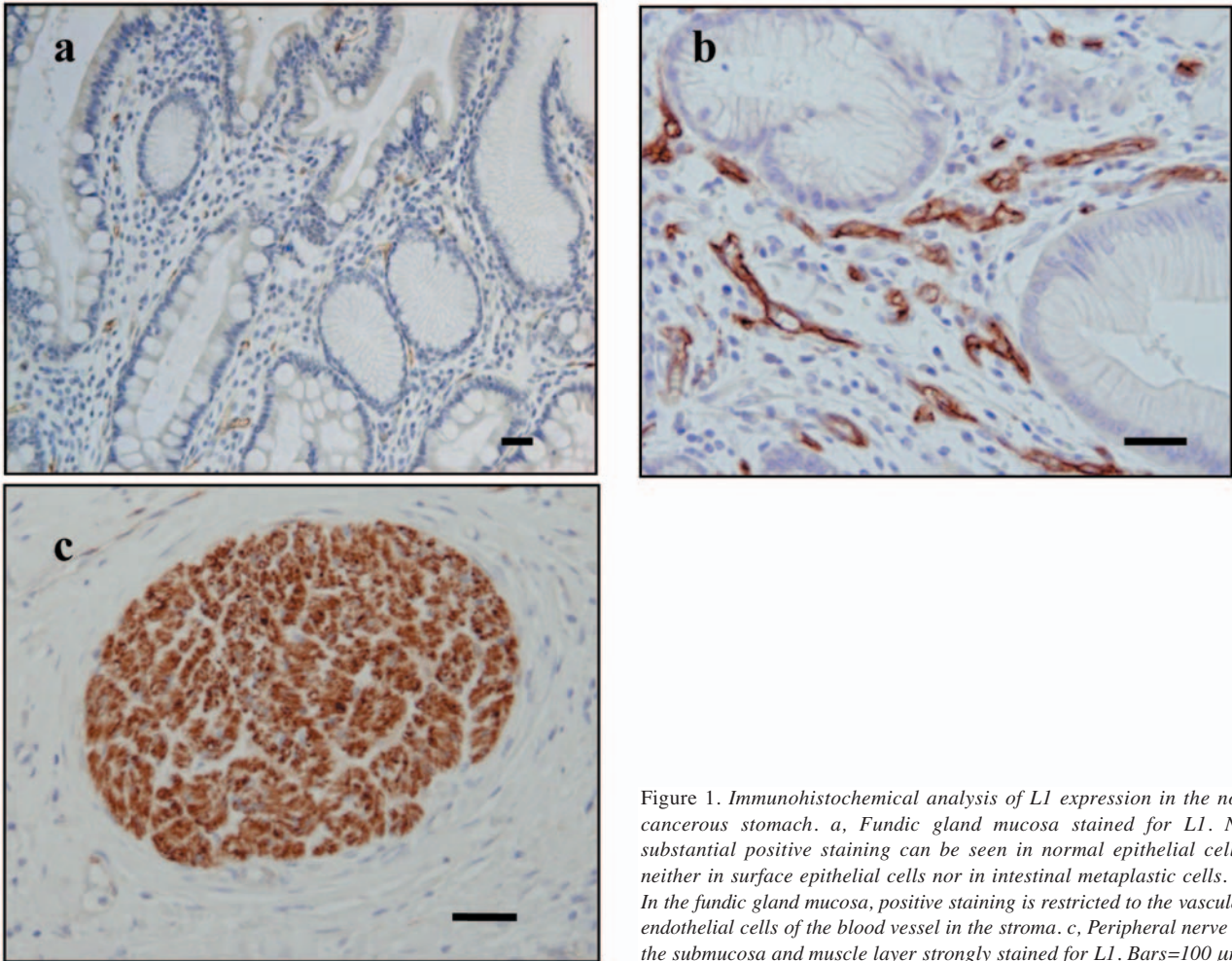*Key Words:* L1, metastasis, gastric cancer, prognostic factor.

Figure 1. *Immunohistochemical analysis of L1 expression in the non cancerous stomach. a, Fundic gland mucosa stained for L1. No substantial positive staining can be seen in normal epithelial cells, neither in surface epithelial cells nor in intestinal metaplastic cells. b, In the fundic gland mucosa, positive staining is restricted to the vascular endothelial cells of the blood vessel in the stroma. c, Peripheral nerve in the submucosa and muscle layer strongly stained for L1. Bars=100 μm.*

Patients and Methods

Patients and specimens. A panel of 72 paraffin-embedded specimens of pT3-stage gastric cancer resected at Department of Gastroenterological Surgery, Aichi Cancer Center, between June 1998 and June 2001 form the basis of this study. In addition, 13 paired samples of primary gastric cancer and corresponding peritoneal metastasis, 8 paired samples of primary gastric cancer and corresponding hepatic metastasis, and 42 samples of colorectal cancer were analyzed. Informed consent had been obtained from all patients regarding use of the specimens for the immunohistochemical studies, and the retrospective analysis has been approved by the institutional review board.

The mean age of the 72 patients in the dataset was 59.5±12.6 years with a male:female ratio of 44:28. Eighteen patients had intestinal-type cancer and 54 had diffuse-type cancer. Sixty-three out of 70 patients had nodal metastasis (information regarding another two patients was unavailable due to the fact that systemic lymphadenectomy had not been performed). Peritoneal deposits were found at laparotomy and histologically confirmed in 19 patients (28%). Cytological examination of the peritoneal washes by Papanicolaou staining was routinely performed and free cancer cells were detected in 23 patients (32%). Four patients had concomitant metastasis to the liver. Gastrectomy with D2 lymphadenectomy was the treatment of choice for all patients who had been considered potentially curable at surgery. None of the patients had received preoperative chemotherapy. Various postoperative therapies were performed at the discretion of the physicians when surgery was deemed non-curative, but adjuvant therapies were not given after R0 resection. All the patients were followed up for at least 5 years (median follow-up time: 2,231 days, range: 1,832-3,289 days, or until death). The follow-up program consisted of interim history, physical examination, hematology and blood chemistry panels including tests for carcinoembryonic antigen (CEA) and carbohydrate antigen (CA) 19-9, and computerized tomography (CT) performed at least once every 6 months. Sites of recurrence were diagnosed based on imaging studies and histological confirmation through biopsy and paracenthesis was performed where possible.

Immunohistochemistry. Histology and immunohistochemistry were performed on sections of 4 μm thickness from formalin-fixed, paraffin-embedded tissues dewaxed through xylene and graded
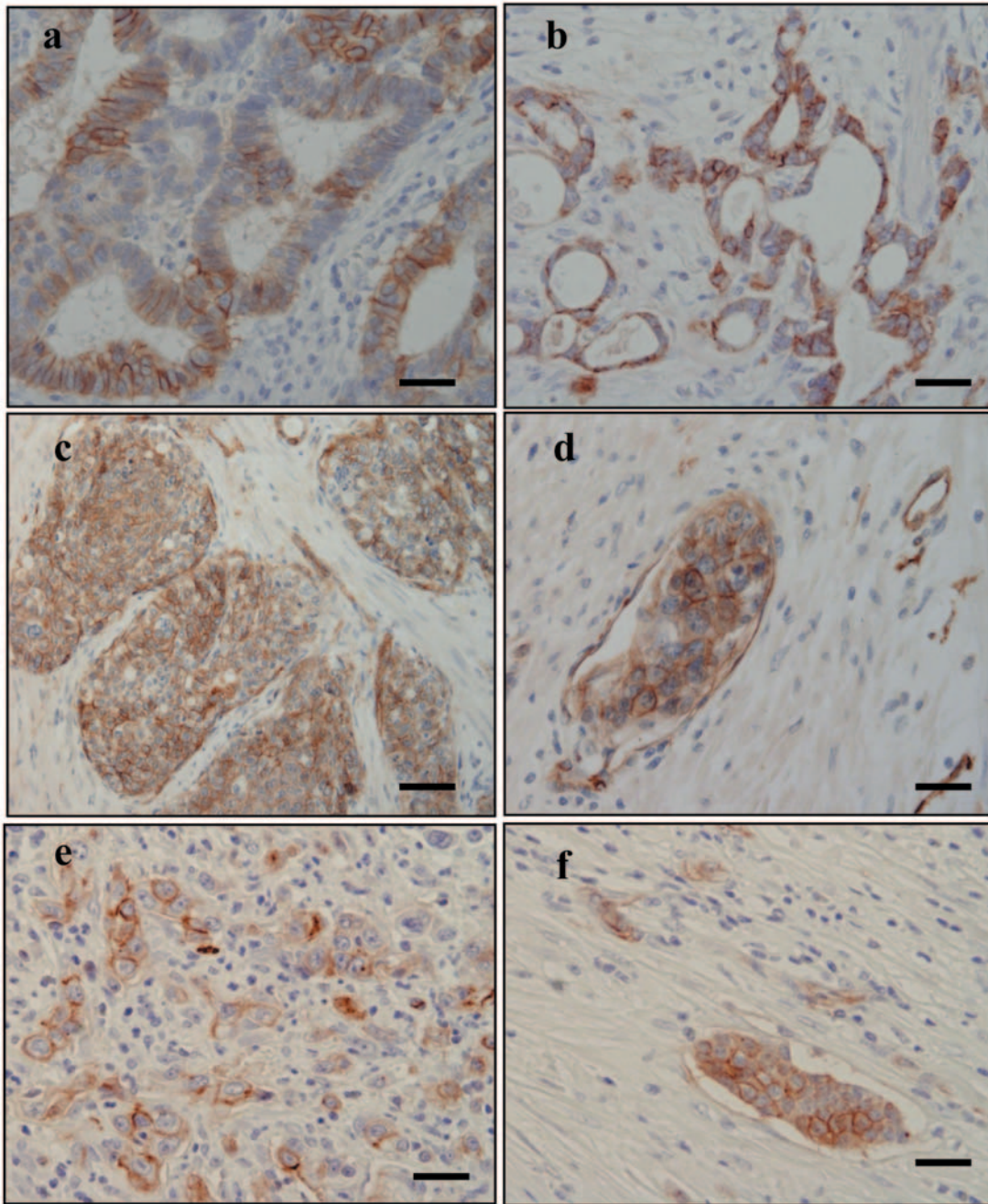
Figure 2. *Immunohistochemical analysis of L1 expression in the primary gastric cancer specimens. a, Well-differentiated adenocarcinoma stained for L1 in the primary tumor. b, Moderately-differentiated adenocarcinoma stained for L1 in the primary tumor. In these tumors, L1 is mainly localized at the cell surface membrane, especially the lateral surface and partly in the cytoplasm. c, Poorly differentiated adenocarcinoma, medullary type, stained for L1 in the primary tumor. d, The same tumor as in c, permeated into the blood vessels strongly stained for L1 in both membrane and cytoplasm. Endothelial cells of the blood vessels also stained positively. e, Poorly differentiated adenocarcinoma, scirrhous type, stained for L1 in the primary tumor. f, The same tumor as in e, permeated into the lymphatic vessels stained positively. Bars=100 μm.*

concentrations of ethanol. For the histology, sections were stained with hematoxylin and eosin. For immunohistochemistry, sections were pretreated for antigen retrieval by microwaving at 98˚C for 10 min (pH 6.0) for L1. These sections were immersed in methanol with 0.3% hydrogen peroxide for 20 min to inactivate endogenous peroxidase activity, followed by normal horse serum for 30 min to block nonspecific reactions. Sections were incubated at 4˚C overnight with mouse monoclonal antibodies against human L1 (1:100 dilution; Thermo Scientific, Fremont, CA, USA) diluted with phosphate-buffered saline (PBS) containing 1% bovine serum albumin. After

Table I. *Association of L1 expression with clinicopathological factors in gastric cancer.*

Variable	L1 Expression		
	–	+	p-Value
Gender			
Male	34	10	
Female	23	5	0.8437
Histology			
Intestinal	10	8	
Diffuse	47	7	0.0120
Nodal metastasis			
Negative	6	1	
Positive	50	13	>0.9999
Concomitant peritoneal metastasis			
Negative	43	10	
Positive	14	5	0.7214
Stage			
II	4	1	
III	23	4	
IV	30	10	0.5923
Peritoneal washing cytology			
Negative	40	9	
Positive	17	6	0.6593
Hepatic metastasis			
No	54	14	
Yes	3	1	>0.9999
Recurrence as peritoneal carcinomatosis (including concomitant metastasis)			
No	25	6	
Yes	32	9	>0.9999
Recurrence to the liver			
No	53	11	
Yes	4	4	0.0905
Recurrence to the bone			
No	52	14	
Yes	5	1	>0.9999

Figure 3. *Survival curves of all 72 pT3-stage gastric cancer patients stratified by L1 expression. The difference between L1-positive cases and L1-negative cases was statistically significant. A survival curve of the 6 cases where L1 staining was observed in the invasion front is superimposed (dotted line).*

Figure 4. *Survival curves of patients who either underwent R2 resection or had recurrence during the 5 years of follow-up, stratified by expression of L1.*

washing with PBS, sections were incubated with biotinylated second antibody for 30 min. Sections were washed again with PBS, then incubated with streptavidin-peroxidase complex (Vectastain ABC kit; Vector, Burlingame, CA, USA) for 60 min. Chromogen was developed with 0.01% diaminobenzidine, and sections were counterstained with hematoxylin. A board-certified pathologist (H.N.) who was blinded to the clinical stage and outcome of the patients evaluated the sections using a scoring system based on percentage of positively stained cells (0, <10% positive cells; 1+, 10-50% positive cells; 2+, >50% positive cells) according to the method of Sato *et al*. (16). The sections were eventually graded as either L1-negative (0) or L1- positive (1+ and 2+). Care was taken to detect any specific immunoreactivity at the invasive front.

Statistical analysis. Chi-square test was performed to evaluate correlation between the expression of L1 and various clinicopathological factors and patterns of disease failure. Survival curves were constructed by the Kaplan-Meier method and the log-rank test was used to evaluate the differences between the curves.
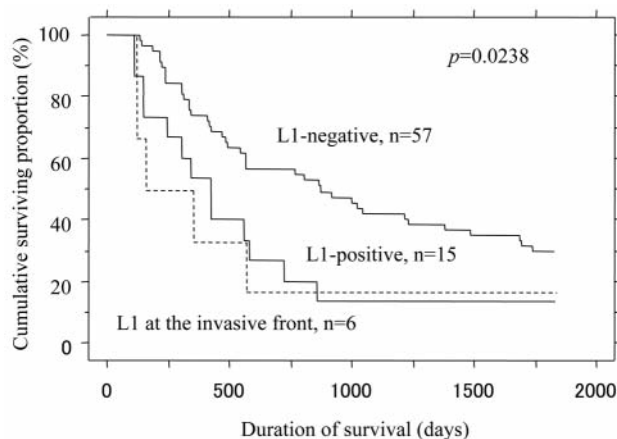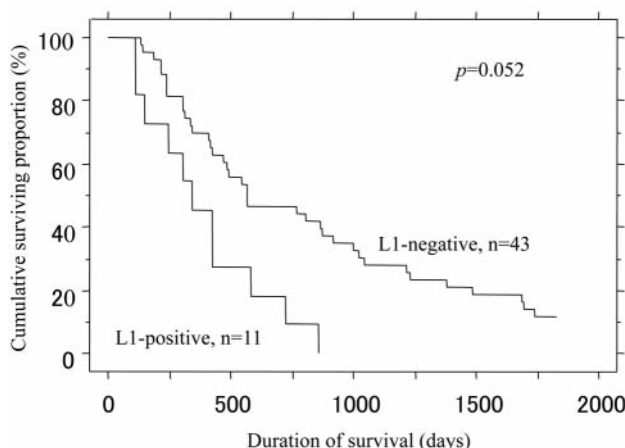
Multivariable analysis was performed with Cox's proportional hazards model with L1 expression, peritoneal deposits at surgery, free cancer cells as evaluated by cytological examination of the peritoneal washes at surgery, and nodal metastasis as covariates.

Results

Outcome and patterns of disease failure. There was no operative mortality. The median survival of all patients was 581 days. Fifty-four out of 72 patients had recurrences during the follow-up. Recurrence as peritoneal carcinomatosis was observed in 41 patients, hepatic metastasis in 8 patients, and bone metastasis in 6 patients. Peritoneal metastasis/recurrence was observed in 76% of all patients with recurrent disease, and was the predominant pattern of failure among this population.
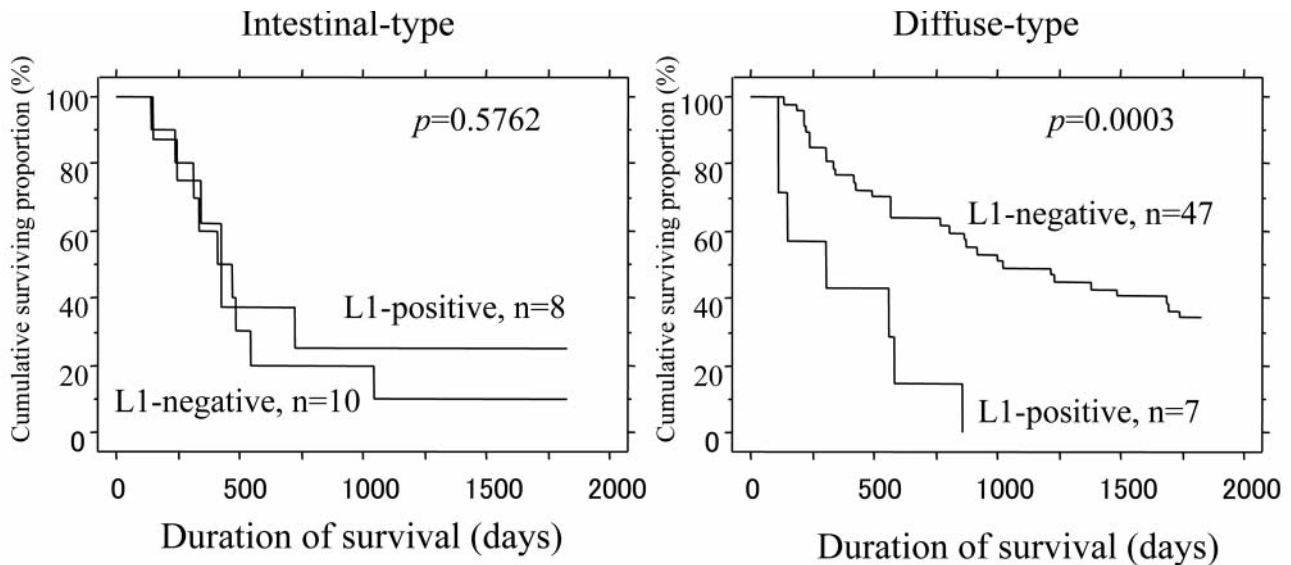
Figure 5. *The difference in survival between patients with L1-positive and those with L1-negative tumors was significant among the diffuse-type, whereas the difference was unclear among the intestinal-type.*

Expression of L1. L1 was not observed in non-cancerous epithelial cells, neither among the pyloric gland epithelial cells, nor the hyperplastic mucosal epithelial cells adjacent to cancer as seen in Figure 1a. In contrast, L1 was detected in the peripheral nerves in the submucosa and muscular layers of the stomach and vascular endothelial cells, but not lymphatic endothelial cells (Figure 1b and 1c). Expression in the neural tissue was used as a positive control, confirming quality of the specimens. L1 was clearly detected in 15 out of 72 specimens (21%). The degree of expression was 1+ in 12 specimens and 2+ in 3, and these will be treated collectively as positive for L1 in the subsequent analyses. L1 was more frequently expressed in the intestinal-type cancer than the diffuse-type (*p*=0.012) (Figure 2, Table I). Expression of L1 was usually limited to the cell surface membrane, particularly the lateral surface, and was suggestive of its involvement in cell to cell adhesion. The cytoplasm was only occasionally and partially stained. Although less frequently, L1-positive cells were observed in diffuse-type cancer, including linitis plastica type cancer. L1-positive tumor thrombi were sometimes observed. In 6 out of 15 L1-positive specimens, overexpression of L1 in the invasion front was observed. Three of these patients had intestinal-type cancer; two of these patients suffered from liver metastasis. Similar immunohistochemical staining was attempted for 44 colorectal cancer specimens to confirm the validity of our immunostaining technique, and surprisingly, cancer cells in the invasive front were stained in all 18 surgical specimens that were L1-positive.

L1 expression was also evaluated in the metastatic lesions. As for peritoneal metastasis, 13 pairs of the primary gastric cancer and corresponding peritoneal metastasis were available for evaluation, of which 10 were diffuse-type cancer. Only 2 primary tumors (one diffuse-type and one intestinal-type) stained positively for L1; L1 was detected in the peritoneal deposits only in the case of diffuse-type cancer. Of the 8 paired specimens of primary cancer and the corresponding hepatic metastasis, only one primary cancer was L1 positive, whereas the liver metastases of this patient and two others stained positively for L1. All three tumors were of the intestinal phenotype.

Association of L1 with metastasis (Table I) and prognosis. No correlation was observed between expression profile of L1 in the primary cancer and presence of concomitant peritoneal metastasis (*p*=0.721), detection of free cancer cells in the peritoneal cavity (*p*=0.659), or development of peritoneal carcinomatosis during the course of follow-up (*p*>0.999). A weak correlation of L1 with recurrence in the liver was suggested, but did not reach a statistical significance (*p*=0.091). L1 was expressed throughout stages II-IV, with no significant difference between the stage groups (*p*=0.592). Nevertheless, survival of patients with L1-expressing cancer was significantly worse than those with non-expressing cancer (*p*=0.024, Figure 3). Multivariable analysis with L1, peritoneal seeding, cytological examination and nodal metastasis as covariates revealed L1 to be barely short of being an independent prognostic factor (hazard ratio 1.94, 95% confidence interval 0.99-3.81, *p*=0.0536). The difference in survival remained nearly significant when compared only among patients who had metastatic/recurrent disease (Figure 4). The difference in survival between patients with L1-

expressing cancer and those with non-expressing cancer was remarkable among the diffuse-type cases whereas no difference was observed among cases of the intestinal-type (Figure 5). There was no long-term survivor among patients with L1-positive diffuse-type cancer. All 7 patients succumbed to peritoneal carcinomatosis with a median survival time only of 309 days. Finally, prognosis of the 6 patients with L1 in the invasion front was even worse, with a median survival time of 149 days (Figure 3).

Discussion

L1 expression was observed in 21% of the pathologically confirmed T3-stage gastric cancer cases. It was statistically more commonly detected in the intestinal-type cancer (8/18, 44%), equivalent to the detection rate of 43% in colorectal cancer specimens evaluated as referential data. This surpassed the percentages for colorectal cancer found in the literature that ranged from 10 to 13% (12, 13). Whether this is due to the ethnic difference, difference in clinical stages or difference in the technique and antibodies used is unclear at this time. In the current study, immunostaining was selected since there was a possibility that only L1 that is localized at the invasive front may be clinically relevant (14). L1 expression at the invasive front was actually confirmed in all 18 cases of L1-positive colorectal cancer, whereas it was observed in only 6 out of 15 L1-positive specimens among the current gastric cancer series. Detection of L1 in the invasive front did implicate aggressive disease and 5 out of the 6 patients died of cancer, two from hepatic, two from peritoneal and one from meningeal recurrences.

All cases evaluated in the current study were of T3-stage and therefore had potential for concomitant peritoneal metastasis or a recurrence as peritoneal carcinoma. While 19 patients had concomitant peritoneal metastasis and a further 22 patients had such recurrences, 31 other patients had no signs of peritoneal disease for 5 years or until death due to other patterns of disease failure. L1 expression of the primary tumor was eventually shown by Chi-square test not to be associated with the finding of concomitant peritoneal deposits, detection of free cancer cells in the peritoneal cavity, or recurrence as peritoneal carcinomatosis during the follow-up. However, since detection of peritoneal disease remains unreliable even through modern imaging studies, the possibility that the 31 patients who had no signs of peritoneal seeding in fact had subclinical peritoneal deposits cannot be dismissed. In addition, a possibility that L1 is expressed transiently in free cancer cells floating in the peritoneal cavity and plays a role in the early phase of peritoneal metastasis formation cannot be denied. Diffuse-type cancer that expressed L1 in general had poor prognosis and all recurrences among this phenotype actually took the form of peritoneal metastases.

On the other hand, there remained a possibility that L1 is associated with hepatic metastasis and recurrence. L1 was shown to be more commonly detected in cases of hepatic disease when compared with those of peritoneal disease. Since hepatic metastasis is not so frequent among gastric cancer patients, however, this study is underpowered to statistically show its association with L1 expression. One can only conclude here that expression of L1 in the primary is not a predictor of any particular pattern of metastasis.

The detection rate of L1 did not significantly correlate with clinical stages at surgery. Surprisingly, however, the expression of L1 indicated poor prognosis among all gastric cancer patients evaluated, particularly among those with diffuse-type cancer. Further *in vitro* studies to identify the role of L1 in gastric cancer progression are ongoing. L1 remained a significant prognostic factor when survival analysis was performed only among patients who either underwent palliative R2 resection or those who eventually had recurrences. Although details are unavailable, it is likely that most of these patients had been treated with chemotherapy. Apart from the speculation that L1 affects several signaling pathways leading to enhanced cell migration and proliferation (17-19), there are lines of evidence suggesting that L1 may have contributed to worsening outcome through its role in protecting cancer cells from chemotherapy-induced apoptosis (20, 21).

To conclude, L1 expression in the primary lesion is a relatively strong prognostic factor for patients with pathologically confirmed T3-stage gastric cancer and may play a role in disease progression, especially of diffuse-type cancer. However, it was not possible to document its role in the formation of peritoneal metastases at this time.

References

1 Pisani P, Parkin DM, Bray F and Ferlay J: Estimates of the worldwide mortality from 25 cancers in 1990. Int J Cancer *83*: 18-29, 1999.

2 Yokota T, Ishiyama S, Saito T, Teshima S, Shimotsuma M and Yamauchi H: Treatment strategy of limited surgery in the treatment guidelines for gastric cancer in Japan. Lancet Oncol *4*: 423-428, 2003.

3 Maehara Y, Hasuda S, Koga T, Tokunaga E, Kakeji Y and Sugimachi K: Postoperative outcome and sites of recurrence in patients following curative resection of gastric cancer. Br J Surg *87*: 353-357, 2000.

4 Yoo CH, Noh SH, Shin DW, Choi SH and Min JS: Recurrence following curative resection for gastric carcinoma. Br J Surg *87*: 236-252, 2000.

5 Kodera Y, Nakanishi H, Ito S, Mochizuki Y, Ohashi N, Yamamura Y, Fujiwara M, Koike M, Tatematsu M and Nakao A: Prognostic significance of intraperitoneal cancer cells in gastric carcinoma: analysis of real-time reverse transcriptase-polymerase chain reaction after 5 years of follow-up. J Am Coll Surg *202*: 231-236, 2006.

6 Raveh S, Gavert N and Ben-Ze'ev A: L1 cell adhesion molecule (L1CAM) in invasive tumors. Cancer Lett 2009, in press

7 Fogel M, Gutwein P, Mechtersheimer S, Riedle S, Stoeck A, Smirnov A, Edler L, Ben-Arie A, Huszar M and Altevogt P: L1 expression as a predictor of progression and survival in patients with uterine and ovarian carcinoma. Lancet *362*: 869-875, 2003.

8 Bao S, Wu Q, Li Z, Sathornsumetee S, Wang H, McLendon RE, Hjelmeland AB and Rich JN: Targeting cancer stem cells through L1CAM suppresses glioma growth. Cancer Res *68*: 6043-6048, 2008.

9 Allory Y, Matduoka Y, Bazille C, Christensen EI, Ronco P, and Debiec H: The L1 cell adhesion molecule is induced in renal cancer cells and correlates with metastasis in clear cell carcinoma. Clin Cancer Res *11*: 1190-1197, 2005.

10 Meier F, Busch S, Gast D, Goeppert A, Altevogt P, Maczey E, Riedle S, Garbe C and Schittek B: The adhesion molecule L1 (CD171) promotes melanoma progression. Int J Cancer *119*: 549-555, 2006.

11 Kaifi JT, Zinnkann U, Yekebas EF, Schurr PG, Reichelt U, Wachowiak R, Fiegel HC, Petri S, Schachner M and Izbicki JR: L1 is a potential marker for poorly-differentiated pancreatic endocrine carcinoma. World J Gastroenterol *12*: 94-98, 2006.

12 Kaifi JT, Reichelt U, Quaas A, Schurr PG, Wachowiak R, Yekebas EF, Strate T, Schneider C, Pantel K, Schachner M, Sauter G and Izbicki JR: L1 is associated with micrometastatic spread and poor outcome in colorectal cancer. Mod Pathol *20*: 1183-1190, 2007.

13 Boo YJ, Park JM, Kim J, Chae YS, Min BW, Um JW and Moon HY: L1 expression as a marker of poor prognosis, tumor progression and short survival in patients with colorectal cancer. Ann Surg Oncol *14*: 1703-1711, 2007.

14 Gavert N, Concacci-Sorrell M, Gast D, Schneider A, Altevogt P, Brabletz T and Ben-Ze'ev A: L1, a novel target of beta-catenin signaling, transforms cells and is expressed at the invasive front of colon cancers. J Cell Biol *168*: 633-642, 2005.

15 Arlt MJ, Novak-Hofer I, Gast D, Gschwend V, Moldenhauer G, Grunberg J, Honer M, Schubiger PA, Altevogt P and Krueger A: Efficient inhibition of intraperitoneal tumor growth and dissemination of human ovarian carcinoma cells in nude mice by anti-L1 cell adhesion molecule monoclonal antibody treatment. Cancer Res *66*: 936-943, 2006.

16 Sato T, Murakumo Y, Hagiwara S, Jijiwa M, Suzuki C, Yatabe Y and Takahashi M: High-level expression of CD109 is frequently detected in lung squamous cell carcinomas. Pathol Int *57*: 719-724, 2007.

17 Gast D, Riedle S, Kiefel H, Muerkoster SS, Schaefer H, Schaefer MK and Altevogt P: The RGD integrin binding site in human L1-CAM is important for nuclear signaling. Exper Cell Res *314*: 2411-2418, 2008.

18 Gast D, Riedle S, Issa Y, Pfeifer M, Beckhove P, Sanderson MP, Arlt M, Moldenhauer G, Fogel M, Krueger A and Altevogt P: The cytoplasmic part of L1-CAM controls growth and gene expression in human tumors that is reversed by therapeutic antibodies. Oncogene *27*: 1281-1289, 2008.

19 Issa Y, Nummer D, Seibel T, Muerkoster SS, Koch M, Schmidz-Winnenthal FH, Galindo L, Weitz J, Beckhove P and Altevogt P: Enhanced L1CAM expression on pancreatic tumor endothelium mediates selective tumor cell transmigration. J Mol Med *87*: 99-112, 2009.

20 Sebens-Muerkoster S, Werbing V, Sipos B, Debus MA, Witt M, Grossmann M, Leisner D, Kotteritzsch J, Kappes H, Kloppel G, Altevogt P, Flosch UR and Schafer H: Drug-induced expression of the cellular adhesion molecule L1CAM confers anti-apoptotic protection and chemoresistance in pancreatic ductal adenocarcinoma cells. Oncogene *26*: 2759-2768, 2007.

21 Sebens-Muerkoster S, Koetteritzsch J, Geismann C, Gast D, Kruse ML, Altevogt P, Foelsch UR and Schaefer J: Alpha5-integrin is crucial for L1CAM-mediated chemoresistance in pancreatic adenocarcinoma. Int J Oncol *34*: 243-253, 2009.