Expression of L1 Cell Adhesion Molecule and Morphologic Features at the Invasive Front of Colorectal Cancer

Yoshiki Kajiwara, MD,1 Hideki Ueno, MD,1 Yojiro Hashiguchi, MD,1 Eiji Shinto, MD,1 Hideyuki Shimazaki, MD,2 Hidetaka Mochizuki, MD,1 and Kazuo Hase, MD1

Key Words: Colorectal cancer; Poorly differentiated component; Solid cancer nest; Tumor budding; Invasive front

Abstract

To obtain the correlation between morphologic features in the invasive fronts of colorectal cancer (CRC) and L1 cell adhesion molecule (L1CAM) expression, 275 CRCs were assessed with L1CAM immunostaining and 29 CRCs were examined for L1CAM messenger RNA (mRNA) expression. Based on immunostaining, the positive rate of L1CAM expression increased according to the grade of tumor budding \((P = .0002)\) and solid cancer nests \((SCNs; P = .0046)\). L1CAM mRNA levels at the invasive front of the tumor were higher than those at the center of the tumor \((median, 3.7\text{-fold})\). The gap of L1CAM mRNA level between the invasive front and the central area was 7.3-fold in tumors having SCN lesions, whereas it was 1.9-fold in tumors having non-SCN lesions \((P = .0004)\). L1CAM expression was correlated with nodal involvement in protein and mRNA levels \((P = .0007\) and \(P = .036\), respectively). Tumor regulation of L1CAM expression is associated with morphologic features at the invasive front in CRC.

The biologic activity of cancer is thought to be more accurately reflected by histologic characteristics at the invasive front than in the central area of tumor. For example, tumor budding observed in the invasive front of the tumor is thought to be associated with the first and essential step in tumor invasion and has been reported as a good index to estimate the aggressiveness of colorectal cancer by many investigators.\(^1\text{-}\text{7}\) Moreover, we showed that the extent of the poorly differentiated component \(\text{(cancer area without a gland structure in the invasive front)}\) was an independent prognosticator.\(^8\) However, the molecular biologic characteristics of these pathologic findings at the invasive front remain unclear.

The L1 cell adhesion molecule \((L1CAM)\) is one of the immunoglobulin superfamily of cell surface proteins.\(^9\text{-}\text{10}\) In an in vitro study, Primiano et al\(^1\text{1}\) reported that the monoclonal antibody against L1CAM strongly inhibited the growth of several tumor cell lines \(\text{(breast, colon, and cervical cancer)}\) but did not inhibit the growth of normal cells. In an in vivo study, Arlt et al\(^1\text{2}\) showed that anti-L1CAM antibodies inhibited the proliferation of the intraperitoneal growth of human ovarian cancer cells in nude mice.

Recent studies show that L1CAM is a potential candidate for therapeutic intervention in colorectal cancer.\(^1\text{3}\text{-}\text{15}\) L1CAM expression in colorectal cancer is known to be correlated with advanced tumor stages and poor prognosis;\(^1\text{6}\text{-}\text{17}\) recently, it has been reported that L1CAM expression is located at the invasive front of the tumor, suggesting that L1CAM may promote the invasiveness of colorectal cancer cells.\(^1\text{3}\text{-}\text{16}\)

However, it is unclear what morphologic features appearing in tumor cells expressed L1CAM at the invasive front. In the present study, we examined the correlation between pathologic parameters of the tumor’s invasive front (such as tumor...
Materials and Methods

Cases

A total of 275 cases of patients who underwent curative resection of advanced colorectal cancer were pathologically reviewed by immunohistochemical analysis. All patients underwent surgery at the Department of Surgery, National Defense Medical College, Saitama, Japan, between 1995 and 1999. The patients comprised 176 men and 99 women, with an average age at the time of surgery of 62.2 years (range, 23-94 years). No patients had received chemotherapy or radiotherapy before surgery.

The expression of L1CAM mRNA was analyzed in a total of 29 cases in which the tumors were dissected by laser microdissection (19 men and 10 women; average age, 64.6 years; range, 47-91 years). The patients underwent surgical resection of primary colorectal cancer between April and September 2008 at our institution.

Written informed consent was obtained from every patient according to institutional regulation, and the study protocol was approved by the National Defense Medical College Ethics Committee.

Parameters

In addition to the conventional clinicopathologic parameters (eg, predominant tumor differentiation, lymphatic invasion, and venous invasion),\textsuperscript{18} the following 2 parameters predominantly observed in the invasive front were assessed: (1) tumor budding: An isolated single cancer cell and a cluster composed of fewer than 5 cancer cells were defined as “budding” foci. The tumor was classified into 4 grades according to the number of budding foci in the area where the foci were observed most intensively (grade I, 0-4; grade II, 5-9; grade III, 10-19; and grade IV, >20).\textsuperscript{3} (2) poorly differentiated component (POR): POR was defined as a region in which the cancer had no glandular formation. Tumors were classified into 3 grades based on the extent of the POR as previously described.\textsuperscript{8} Briefly, grade III was used for tumors in which the POR fully occupied a ×400 microscopic field. For tumors having a smaller POR, solid cancer nests (SCNs) composed of 5 or more cancer cells without gland structure\textsuperscript{Image 1} were counted in a microscopic field of ×40 in which SCNs were observed most intensively. Tumors with 10 or more SCNs were classified as grade II and those with fewer than 10 SCNs as grade I.

Judgments regarding the 2 parameters were made by 1 of us (H.U.) with no information about clinicopathologic data, including L1CAM status or the patient’s prognostic outcome.

Immunohistochemical Staining of L1CAM

For the study, 4-μm-thick sections were cut from formalin-fixed, paraffin-embedded samples. Heat-induced autolysis antigen retrieval was performed at 120°C for 10 minutes with citrate buffer (pH 6.0). Peroxidase blocking (3% hydrogen peroxide in methanol) was performed for 10 minutes, and nonspecific binding was blocked with normal horse serum. Sections were incubated overnight with anti-human L1CAM antibody (prediluted, mouse monoclonal antibody SPM275, Spring Bioscience, Fremont, CA) at 4°C. Specimens were reacted with peroxidase-labeled streptavidin (PK-8800, Vector Laboratories, Burlingame, CA). Reaction products were visualized with diaminobenzidine (SK-4100, Vector Laboratories), and slides were then counterstained with hematoxylin. Immunohistochemical slides were evaluated by 1 of us (Y.K.) who had no knowledge of the clinicopathologic data. The tumor was judged to be L1CAM+ when more than 30% of tumor cells had an immunoreaction for L1CAM (Image 1).

Laser Microdissection

At the time of surgery, 1 section line including the invasive front of the tumor was immediately cut from the resected colorectal specimen by pathologists. The section was embedded in Tissue Tek OCT medium (Sakura, Tokyo, Japan), frozen in liquid nitrogen, and kept at –80°C. The specimens were cut into serial sections with a thickness of 10 μm, and the sections were fixed in 5% acetic acid in ethanol for 3 minutes. The sections were incubated with 0.05% toluidine blue solution, pH 7.0, (Wako Pure Chemical Industries, Osaka, Japan) for 30 seconds. The sections were air dried, and then cancer cells at the center of the tumor and at the invasive front, respectively, were microdissected with a laser microdissection system (Leica Microsystems, Wetzlar, Germany). When the tumor had SCN lesions, the cancer cells at the SCN lesions were selectively harvested as invasive front specimens. In addition, tumor specimens of non-SCN lesions at the invasive front of the tumor were also microdissected from the same tumor to compare the L1CAM status between the SCN and non-SCN parts.

Total RNA Extraction and First-Strand Complementary DNA Synthesis

Total RNA was isolated by using an RNeasy mini kit (Qiagen, Tokyo, Japan) following the manufacturer’s instructions, and all samples were treated with RNase-free DNase set (Qiagen). The RNA quality control and quantification were...
were considered to indicate significance. Statistical calculations by using the Wilcoxon signed rank test. Lesions and non-SCN lesions in the same tumors was assessed between the L1CAM mRNA levels of cancer cells at SCN assessed by using the Mann-Whitney SCN lesions (or nodal involvement), the mRNA levels were the invasive front cancer cells in tumors with and without the Fisher exact test. To compare L1CAM mRNA levels of cancer cells at SCN, the stability of each transcript and sequentially removes the least stable transcript until the 3 most stable transcripts remain. The following primers (Perfect Real Time Support System, Takara Bio) were used for quantitative reverse transcriptase–polymerase chain reaction (RT-PCR): L1CAM, forward, 5'-CAAGATCCAGGCCGTCAACA-3' and reverse, 5'-CAGCACGGCACTTGAAGTTGAG-3'; PGK1, forward, 5'-GACAGGTGTGATGGAATGCGCAAGA-3' and reverse, 5'-TGCTTTCAAGGACACAGTCCA-3'; HPRT1, forward, 5'-GCCAGTTATATCCAAAGATGGTCAA-3' and reverse, 5'-TGCTTTCAAGGACACAGTCCA-3'; and RPLP2, forward, 5'-TGACACCGTGGTATCAGAG-3' and reverse, 5'-CTGGGCAATGACGTCTTCAA-3'. SYBR green real-time PCR was performed using the Thermal Cycler Dice Real-Time System TP800 (Takara Bio), and the complementary DNA from the dissected colorectal cancer cells. PCR conditions were as follows: 30 seconds at 95°C for the initial denaturation and then 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. All reactions were run in duplicate. A melting curve was constructed for each primer pair to confirm product specificity. Real-time PCR data were analyzed by using Multiplate RQ software (Takara Bio), which is able to treat multiple reference genes. Relative gene expression was determined by a comparative Ct method. Statistical Analysis The association between L1CAM expression and clinico-pathologic features was analyzed by using the χ² test or the Fisher exact test. To compare L1CAM mRNA levels of the invasive front cancer cells in tumors with and without SCN lesions (or nodal involvement), the mRNA levels were assessed by using the Mann-Whitney U test. The correlation between the L1CAM mRNA levels of cancer cells at SCN lesions and non-SCN lesions in the same tumors was assessed by using the Wilcoxon signed rank test. P values less than .05 were considered to indicate significance. Statistical calculations were performed using Stat View, version 5.0 software (SAS Institute, Cary, NC) and the SPSS software package (SPSS, Chicago, IL).

Results

Correlation Between Immunohistochemical Expression of L1CAM and Conventional Clinicopathologic Parameters

Positive expression of L1CAM was found in 95 (34.5%) of 275 cases. L1CAM immunoreaction was mainly observed in the cell surface membrane [Image 1BI]. L1CAM immunoreactivity was detected in random areas, mainly at the tumor invasive front.

The relationship between L1CAM immunoreactivity and conventional clinicopathologic characteristics in the total series is summarized in [Table 1]. L1CAM expression was not related to depth of tumor invasion, but it was closely related to nodal status (P = .0007). In addition, L1CAM expression was correlated with high-grade lymphatic invasion (P = .037) and venous invasion (P = .012).

Correlation Between Immunohistochemical Expression of L1CAM and Pathologic Parameters of the Invasive Front of the Tumor

Cases were classified according to the grade of tumor budding as follows: grade I, 115 cases (41.8%); grade II, 54 cases (19.6%); grade III, 60 cases (21.8%); and grade IV, 46 cases (16.7%). With regard to the extent of POR, cases were divided into 3 grades: grade I, 32 cases (11.6%); grade II, 138 cases (50.2%); and grade III, 105 cases (38.2%). L1CAM expression was significantly associated with POR grade (P = .0046) and tumor budding (P = .0002). The positive rate of L1CAM expression increased according to the grade of tumor budding and POR (Table 1).

Up-regulation of L1CAM mRNA in the Invasive Front of the Tumor

L1CAM mRNA levels in the invasive front of the tumor were higher than those at the center of tumor in all but 2 of the 29 tumors. Overall, the L1CAM mRNA level was significantly higher at the invasive front than at the center of the tumor (median, 3.67-fold; range, 0.74- to 226-fold; P = .0015) [Figure 11]. Regarding L1CAM mRNA levels of cancer cells at the center of tumor as the standard, L1CAM mRNA levels of invasive front cancer cells were found to be significantly more up-regulated in patients with nodal involvement (median, 6.30-fold; range, 1.01- to 226-fold) than in patients without nodal involvement (median, 2.21-fold; range, 0.74- to 19.4-fold; P = .036) [Figure 21].
Correlation of L1CAM mRNA Expression and Poorly Differentiated Component in the Invasive Front of the Tumor

With regard to the gap of the L1CAM mRNA level between cancer cells at the invasive front and those in the central area, it was 7.3-fold (range, 1.01- to 226-fold) in 13 SCN-positive tumors, whereas it was 1.9-fold (range, 0.74- to 7.0-fold) in 16 SCN-negative tumors (P = .0004) [Figure 3].

Furthermore, a comparison of L1CAM mRNA expression was performed between SCN and non-SCN lesions in the same tumor. L1CAM mRNA expression levels of SCN lesions were consistently higher than those of non-SCN lesions in all SCN-positive cases (median mRNA expression ratio [SCN lesion/non-SCN lesion] 2.56; range, 1.47- to 39.2-fold) (P = .0015) [Figure 4].

Discussed

Several investigators have revealed that cancer cells that express L1CAM present active motility and increase scattering in the in vitro studies.13,20,21 In the present study, we examined surgical specimens of colorectal cancer and confirmed their findings, ie, there was a significant correlation between the morphologic features of the tumor at the invasive front (such as tumor budding and extent of POR) and L1CAM expression. Gavert et al13,14 reported that L1CAM is localized at the invasive front of the colon cancer tissue that expresses nuclear β-catenin, together with the metalloprotease ADAM10, which is involved in the cleavage and shedding of the L1 extracellular domain. We also confirmed that the level of mRNA expression of L1CAM increased in tumor cells at the invasive front compared with tumor cells at the center of the tumor.
A few studies about immunohistochemical L1CAM expression in clinical specimens of colorectal cancer have been reported previously. Boo et al. demonstrated that L1CAM expression was found in 10.9% of the 138 colorectal cancers and was correlated with advanced tumor stages, distant metastasis, and poor survival. Kaifi et al. showed that L1CAM expression was observed in 13% of the 375 primary colorectal cancers and was associated with a poor outcome and micrometastases in the bone marrow and lymph nodes. They used a tissue microarray analysis that evaluates a number of cases simultaneously with only small pieces of tissue. Based on our observation, this method seems to have underestimated L1CAM expression. Because intratumoral heterogeneity of immunoreactivity is observed in L1CAM staining and L1CAM is often located in cancer cells adjacent to stroma, a tissue microarray study for L1CAM might include some degree of false-negative results. In the present study, we evaluated the whole area of 1 section slide, and the L1CAM expression rate was 34.5%, which is much higher than that of both previous studies.

In regard to correlation with conventional clinicopathologic parameters, including predominant tumor differentiation and lymphovascular invasion, L1CAM expression was particularly correlated with nodal involvement in our study. In addition, we ascertained that the extent of up-regulation of L1CAM mRNA expression in cancer cells at the invasive front was significantly related to the status of nodal involvement. These results suggest that L1CAM has a strong impact on lymphatic spread in colorectal cancer. In contrast, there is some controversy as to the relationship between L1CAM expression and distant metastasis. Although Boo et al. reported a strong correlation between L1CAM and distant metastasis, Kaifi et al. showed that none of their 86 patients with distant metastasis had L1CAM-positive tumors. In our study, we found no significant relation between L1CAM expression and distant metastasis.

The immunohistochemical expression of L1CAM was strongly correlated with 2 pathologic parameters (ie, tumor...
Anatomic Pathology / Original Article

**Figure 2** Relative L1 cell adhesion molecule (L1CAM) messenger RNA (mRNA) expression levels in the cancer cells at the invasive front with and without nodal involvement in comparison with levels in cancer cells at the center of the tumor. Y-axis, fold change compared with mRNA expression in the cancer cells at the center of tumor. Boxes, first and third quartiles (median inside); bars, 10th and 90th percentiles. $P = .036$.

**Figure 3** Relative L1 cell adhesion molecule (L1CAM) messenger RNA (mRNA) expression levels in the cancer cells at the invasive front without solid cancer nests (SCNs) and with SCNs in comparison with levels in the cancer cells at the center of the tumor. Y-axis, fold change compared with mRNA expression in the cancer cells at the center of tumor. Boxes, first and third quartiles (median inside); bars, 10th and 90th percentiles. $P = .004$.

**Figure 4** Relative L1 cell adhesion molecule (L1CAM) messenger RNA (mRNA) expression level compared with the L1CAM mRNA expression in the center of the tumor in paired solid cancer nest (SCN) and non-SCN lesions from 13 cases having both SCN and non-SCN lesions. Y-axis, fold change compared with mRNA expression in the cancer cells at the center of tumor. $P = .0015$.

budding and extent of POR) in the invasive front of the tumor. Tumor budding, which is defined as an isolated single cancer cell or a cluster composed of fewer than 5 cancer cells in the invasive front of tumors, is a good semiquantitative index to estimate the aggressiveness of colorectal cancer.\(^2\)\(^3\) Ueno et al\(^3\) reported that high-grade (grade III or IV) tumor budding in patients with rectal cancer was associated with a lower 5-year survival rate than patients with low-grade (grade I or II) tumor budding. In the present study, the positive rate of L1CAM expression in high-grade tumor budding cases was 54.7%, which was significantly higher than the rate of cases with low-grade tumor budding (25.4%). In an in vitro study, the expression of L1CAM was compared in sparse cell cultures (that mimic the invasive phenotype, such as tumor budding) and dense cell cultures of colorectal cancer cell lines, and the sparse cells expressed a higher level of L1CAM mRNA and protein than did dense cells.\(^1\)\(^3\) Based on our results, these phenomena in tumor budding and sparse cell cultures are thought to be related.

To evaluate the meaning of poorly differentiated cancer clusters in the invasive front, we previously established a new grading system based on the extent of the POR.\(^8\) The POR was newly defined as a region where a tumor has no glandular formation and is graded according to the amount of SCNs (composed of ≥5 cancer cells without gland structure [Image 1C]) in expectation of its potential contribution to objective tumor grading. Patients with advanced colorectal
cancer classified as grade I demonstrate a very favorable prognosis. In the present study, we showed that the positive rate of L1CAM expression increased according to the grade of POR. Moreover, based on the real-time RT-PCR analysis, we confirmed that the level of mRNA expression of L1CAM was much higher in the cancer cells of SCN lesions than in those with gland structures. The up-regulation of L1CAM in the invasive front of the tumor might be the essential characteristic of the POR in colorectal cancer.

Our study revealed that L1CAM expression is related to morphologic features in the invasive front of colorectal cancer. L1CAM could have an important role in forming a particular morphologic feature at the invasive front region associated with the aggressiveness of the tumor, especially lymphogenous metastasis.

From the Departments of Surgery and Laboratory Medicine (Pathology), National Defense Medical College, Saitama, Japan.

Address reprint requests to Dr Kajiwara: Dept of Surgery, National Defense Medical College, 3-2, Namiki, Tokorozawa, Saitama 359-8513, Japan.

References