Original Article
L1CAM is involved in lymph node metastasis via ERK1/2 signaling in colorectal cancer

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Abstract: L1-cell adhesion molecule (L1CAM, L1) belongs to the immunoglobulin superfamily and was originally found to play a role in nerve cells. Recently, the expression and prognostic value of L1 has been established in several cancers, including colorectal cancer (CRC). However, its association with lymph node metastasis in CRC and the mechanisms underlying its effects remain unclear. In this study, we evaluated the L1 transcript levels in CRC (n=12) and normal intestinal tissues (n=15) by qRT-PCR. Western blotting was used to evaluate L1 and pERK1/2 expression levels. Immunohistochemistry was performed to evaluate the relationship between L1 and pERK1/2 in CRC tissues with different levels of differentiation. The mRNA expression levels in CRC tissues were significantly higher compared to normal intestinal tissues. Western blotting demonstrated that both L1 and pERK1/2 levels were higher in CRC than in normal tissues. Immunohistochemistry confirmed that L1 and pERK1/2 levels in adenomas with lymph node metastasis were significantly higher than in poorly and well-differentiated adenomas, indicating that L1 and pERK1/2 levels correlated with CRC lymph node metastasis. In conclusion, L1 and pERK1/2 were significantly up-regulated in CRC tissues and lymph node metastasis may occur via the L1CAM-mediated ERK pathway in CRC.

Keywords: L1, lymph node metastatic, ERK1/2, colorectal cancer

Introduction

Colorectal cancer (CRC) is a common malignancy worldwide, especially in Western countries. Necropsy studies have reported a prevalence of about 35% in Europe and the USA, with lower rates (10-15%) in Asia and Africa. However, accurate estimates of morbidity are difficult to obtain in symptom-free and unselected populations [1]. Owing to the rapid development of the Chinese economy and dietary changes, the incidence of CRC has rapidly increased in China. Metastasis accounts for about 90% of all CRC-related deaths. In general, advanced CRC may metastasize to many organs, including the lymph nodes, peritoneum, lungs, and liver. Notably, lymph node metastasis is an invaluable prognostic factor in almost all cancer types [2, 3]. Thus, it is of great importance to explore the molecular mechanisms underlying lymph node metastasis in CRC.

The L1 cell adhesion molecule (L1CAM, L1) is a 200-220 kD transmembrane glycoprotein composed of six immunoglobulin-like domains, five fibronectin-type III domains, a transmembrane stretch, and a short cytoplasmic tail [4]. It was originally identified as a neural cell adhesion molecule in the central nervous system, with an important role in initiating cerebellar cell migration and neurite outgrowth [5]. Recently, L1CAM has been identified in other cell types, including intestinal cells, epithelial cells of the urogenital tract, lymphoid cells, and reticular fibroblasts [6-8]. L1CAM has been identified in a variety of tumor types and is correlated with poor prognosis and metastasis. However, it is not clear whether L1CAM is associated with lymph node metastasis in patients with CRC.

ERK signaling is important for regulating diverse cellular processes, such as proliferation, survival, differentiation, motility, and tumor progression. It has been confirmed that L1CAM

can interact with transmembrane binding partners or ECM receptors, resulting in the activation of the ERK pathway and ERK-dependent genes [9]. However, the role of L1CAM-mediated ERK pathways in lymph node metastasis in CRC has not been examined.

In the present study, we detected the expression of L1CAM in the lymph nodes of patients with CRC and further examined the activation of the ERK1/2 pathway. These results improved our understanding of the L1CAM role in CRC.

Materials and methods

Cell lines and culture conditions

The human colon cancer cell lines, HCT116 and SW620, were obtained from the American Type Culture Collection (ATCC, Rockville, USA). Cell lines were maintained and grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere of 5% CO_2 and 95% air.

Clinical samples

This study was approved by the Ethics Committee of Zhejiang Provincial People's Hospital in Hangzhou, China. Written informed consent was obtained from all patients for the use of resected samples.

Migration assay

Transwell chambers with 4.5-µm pores were obtained from Corning. Cells were harvested and re-suspended in DMEM with 2% FBS at a concentration of $2×10^5$ cells in 1 mL, and then seeded into the upper chamber of a 12-well plate. The lower chambers were filled with 2 mL DMEM containing 10% FBS. Next, cells were incubated for 18 h. At the end of the experiment, cells that migrated into the reverse side of the transwell membrane were fixed with methanol, stained with Giemsa stain solution, and counted under an inverted light microscope.

qRT-PCR

Quantitative real-time PCR (qRT-PCR) with specific primers (*L1*-F and *L1*-R; *GAPDH*-F and *GAPDH*-R) in Table S1 was performed to determine the relative mRNA levels of *L1*. Meanwhile, the relative mRNA levels of *matrix metalloproteinase 2* (*MMP2*), *matrix metalloproteinase 13* (*MMP13*), *integrin beta-3* (*ITGB3*) and *FOS-related antigen 1* (*FRA1*) in SW620 cells with U0126 were carried out with primers listed in Table S1. Total RNAs were isolated using TRIzol reagent (Invitrogen, Shanghai, China) from CRC, normal intestinal tissues, and SW620 cells according to the manufacturer's instructions. All PCRs were performed in a total volume of 10 µL using the SYBR Premix Ex Taq Kit (Takara Bio, Shanghai, China). The amplification conditions for qRT-PCR were as follows: one cycle at 95°C for 30 s, followed by 45 cycles of 95°C for 5 s and 60°C for 20 s, and a single cycle of 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method with *GAPDH* for normalization. Each sample was evaluated in triplicate and measurements were repeated independently at least thrice. An average threshold (Ct) was retrieved for data analysis.

Western blotting

Tissues were added to the cold lysis buffer (150 mM NaCl, 1% NP-40, 50 mMTris-HCl, pH 8.0, supplemented with a complete set of protease inhibitors) (Roche, Basel, Switzerland). Samples were homogenized and incubated for 30 min on ice. Cell lysates were centrifuged at 13,000×*g* for 10 min at 4°C. After the supernatants were collected and quantified, whole protein extracts were added to Laemmli loading buffer and incubated at 99°C for 10 min. Samples (20 µg) were separated by 12% SDS-PAGE under reducing conditions. After blotting onto PVDF membranes, 5% BSA was used for blocking, and the membranes were incubated with anti-*L1* primary antibodies (1:1000; R&D, Minneapolis, MN, USA), followed by a secondary HRP-conjugated IgGAb (1:8,000). Immuno-reactive proteins were visualized using a chemiluminescent immunodetection system (ChemiDoc XRS). Primary antibodies of pERK1/2 (1:1000; R&D, Minneapolis, MN, USA), ITGB3 (1:1000; Abcam, Hongkong, China), MMP2 (1:1000; Abcam, Hongkong, China), MMP13 (1:3000; Abcam, Hongkong, China), and FRA1 (1:1000; Abcam, Hongkong, China) were applied using the same method described above. ImageJ 2× was employed to analyze the grayscale values obtained by western blotting.
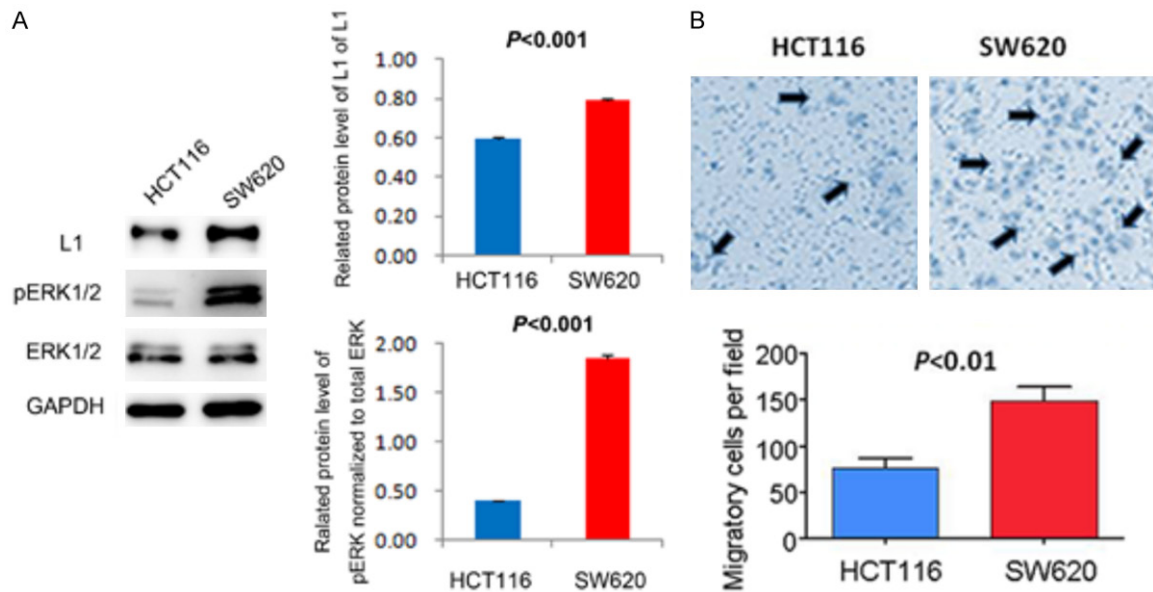
Figure 1. L1 and pERK1/2 expression in colorectal cancer cells and correlated with high motility. A. The L1, pERK1/2, and ERK1/2 protein levels were determined by western blotting, GAPDH was adopted as the loading control. The related protein levels of L1 and pERK1/2 in metastatic (SW620) cells are higher than in non-metastatic (HCT116) cells ($P<0.001$, $P<0.001$, n=3). B. Cell migration was performed using 12-well inserts from Corning. The number of migrated cells was quantified after 18 h of incubation. The data are shown as the mean number of migrated cells per visual field. Colorectal cancer metastasis cells (SW620) demonstrate significantly better migration ability than non-metastatic cells (HCT116) ($P<0.01$). Grayscale analysis was employed with Image J 2×.

Immunofluorescence staining

Intestinal tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and analyzed histologically. The remaining sections were blocked with 5% goat serum at 37°C for 1 h and then stained with primary Abs (anti-L1 or anti-pERK1/2). After washing, the intestinal paraffin slices were incubated with an HRP-conjugated anti-rabbit/mouse IgGAb (1:8000) at 37°C for 1 h. Finally, the sections were incubated with DAB peroxidase substrate (Sigma, St. Louis, MO, USA). The staining results were observed under a light microscope (Olympus BX51, Tokyo, Japan). Image J 2× was employed to analyze the grayscale values.

Statistical analysis

SPSS 17.0 (IBM, Chicago, IL, USA) was used for statistical analyses and t-tests were performed to determine the significance. All data are presented as means ± standard deviation (SD). Statistical differences between two groups were evaluated by Student's *t*-tests. Statistical significance was defined as $P<0.05$.

Results

L1 expression increased in CRC cells and correlated with high motility

We first assessed L1 levels in colon cancer cells and the results indicated that L1 expression was higher in metastatic (SW620) than in non-metastatic (HCT116) cells ($P<0.001$) (**Figure 1A**). In accordance with the unregulated L1 expression, phosphorylated ERK1/2 (pERK1/2) expression was increased in SW-620 cells ($P<0.001$) (**Figure 1A**). In the transwell cell migration assay, 12-well inserts from Corning were used. Cell migration was performed as described above. The number of migrated cells could be quantified by counting underneath a microscope or in the pictures obtained. As shown in **Figure 1B**, there was a 2-fold difference in the SW620 cells migrating toward the chemo-attractant in comparison to HCT116 cells ($P<0.01$).

pERK1/2 expression contributed to CRC cell migration

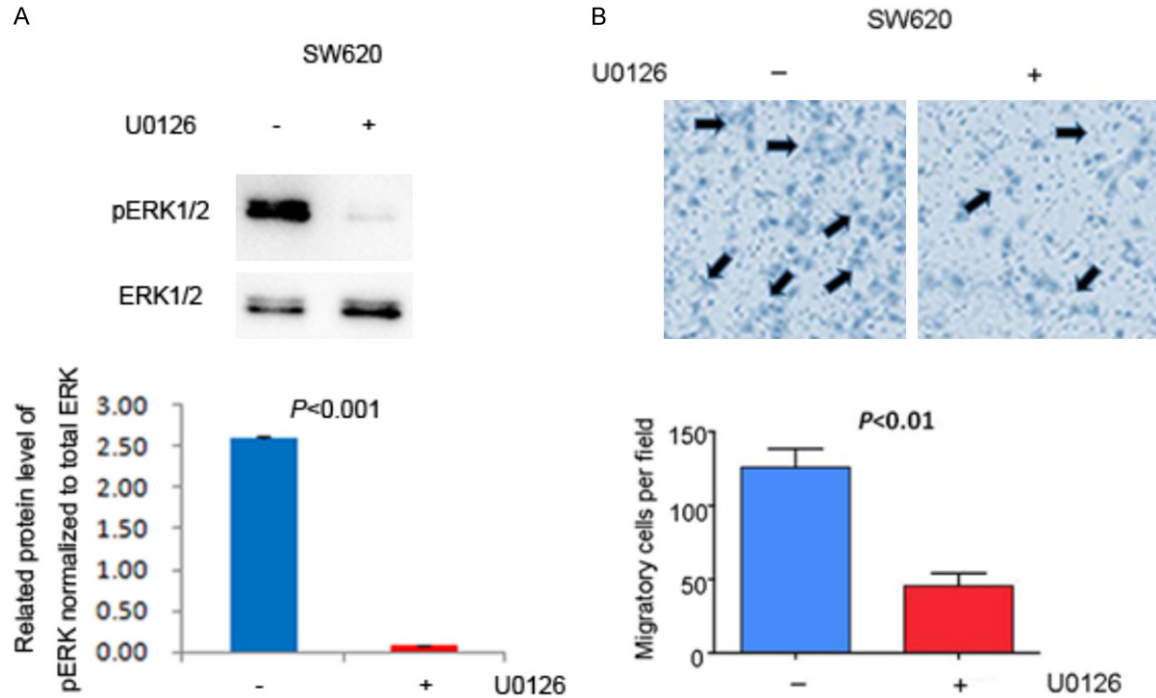Given the involvement of pERK1/2 in promoting cell proliferation and migration, we treated

Figure 2. pERK1/2 inhibition reduces colorectal cancer cell migration. A. SW620 cells were treated with a MEK/ERK kinase 1/2 pharmacologic inhibitor (U0126). Western blotting was performed to determine the protein level of pERK1/2 and ERK1/2 in SW620 cells. The results indicate that U0126 can significantly inhibit the expression of pERK1/2 ($P<0.001$, n=3). B. A significant reduction in migration is observed following U0126 administration. The transwell assay was performed to detect the migration of SW620 cells in culture medium with U0126. The cells in the lower chamber were counted and demonstrate the inhibited migration ability of cells in the U0126 medium ($P<0.01$). Grayscale analysis was employed with Image J 2×.

SW620 cells with a MEK/ERK kinase 1/2 pharmacologic inhibitor (U0126) which blocks pERK1/2 activity ($P<0.001$) (**Figure 2A**). SW-620 cells treated with U0126 demonstrated a significant reduction in migration (~60%) compared to cells treated with DMSO ($P<0.01$) (**Figure 2B**).

L1 overexpression was associated with increased pERK1/2 expression in primary human CRC

The *L1* transcript levels in primary CRC and normal colorectal tissues were evaluated by qRT-PCR. *L1* levels in CRC tissues were higher than those in normal intestinal tissues ($P<0.01$) (**Figure 3A**). Western blotting and grayscale analyses were performed to evaluate the *L1* and *pERK1/2* expression levels. L1 levels in CRC tissues were significantly higher than those detected in normal intestinal tissues ($P<0.001$) (**Figure 3B**). Additionally, pERK1/2 expression levels in CRC were up-regulated ($P<0.001$) (**Figure 3C**).

Correlations between L1 and pERK1/2 and lymph node metastasis

Poorly differentiated, well-differentiated, and lymph node metastatic adenoma tissues were subjected to paraffin sectioning and immuno-fluorescence staining. L1 and pERK1/2 expression were detected in these three tissues with various levels of differentiation. However, the expression levels of L1 ($P<0.01$, $P<0.01$) (**Figure 4**) and pERK1/2 ($P<0.001$, $P<0.001$) (**Figure 5**) in adenocarcinoma tissues with lymph node metastasis were significantly higher than in both poorly differentiated and well-differentiated adenoma tissues. Moreover, a linear relationship between L1 levels and pERK1/2 activity was observed (**Figure 6**).

High expression of ERK-dependent genes in CRC

A series of experiments were performed to further elucidate the mechanisms involved in the tumor invasion and dissemination mediated by
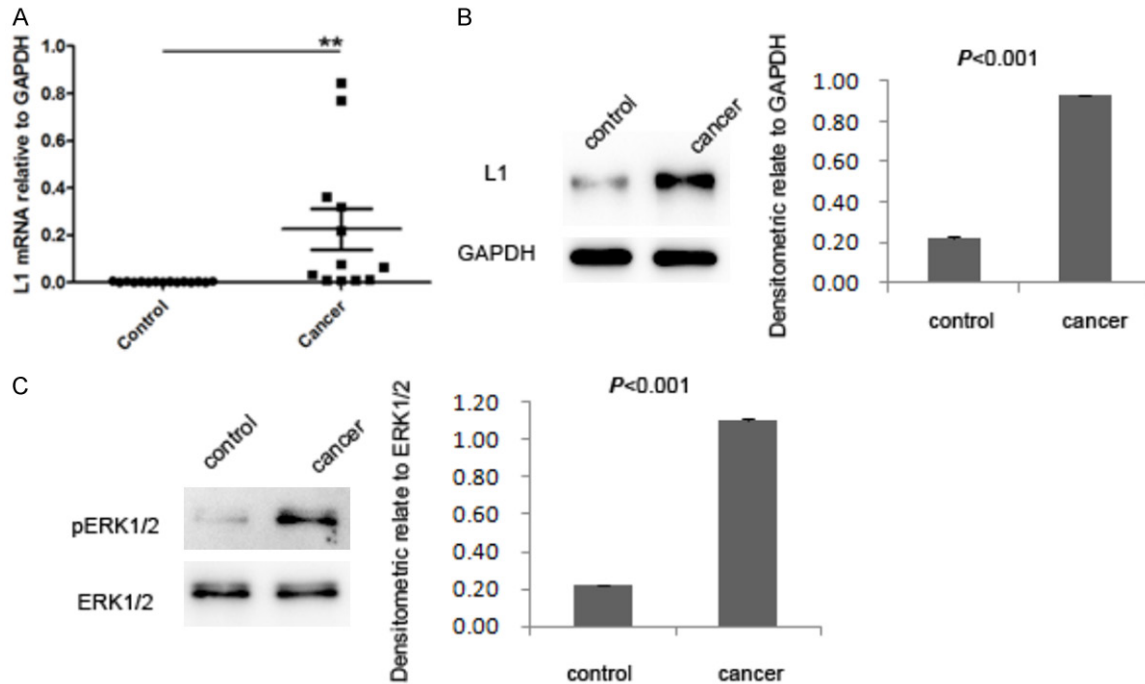
Figure 3. L1 overexpression is associated with increased pERK1/2 expression in primary human colorectal cancer. A. Relative mRNA levels of *L1* in colorectal cancer and normal intestinal tissues. The abundance of *L1* transcripts was quantified by qRT-PCR in colorectal cancer (n=12) and normal tissues (n=15). All gene expression levels were normalized to the *GAPDH*. Notably, the mRNA expression level in colorectal cancer tissues is higher than in normal intestinal tissues (**$P<0.01$). B. L1 expression analysis by western blotting. A GAPDH antibody was used as an internal reference. The grayscale value indicates a significantly higher protein level in colorectal cancer than normal intestinal tissues ($P<0.001$, n=4). C. The expression levels of pERK1/2 and ERK1/2 in colorectal cancer and normal intestinal tissues were detected by western blotting. Like L1, pERK1/2 expression is higher in colorectal cancer than normal intestinal tissues ($P<0.001$, n=4). Grayscale analysis was employed with Image J 2×.

ERK1/2 in CRC. The protein levels of MMP2, MMP13, ITGB3, and FRA1 detected by western blotting were increased in CRC (cancer) tissues compared to normal intestinal (control) tissues ($P<0.001$, $P<0.001$, $P<0.001$, $P<0.001$) (Figure S1). Subsequently, the mRNA expression levels of these genes were determined by qRT-PCR. The blockage of ERK1/2 phosphorylation by treatment with a small chemical inhibitor (U0126) decreased the expression of *MMP2*, *MMP13*, *ITGB3*, and *FRA1* in CRC cells ($P<0.05$, $P<0.05$, $P<0.05$, $P<0.05$) (Figure S2).

Discussion

CRC is a serious disease and the fourth most fatal cancer in the world after lung, liver, and stomach cancers. The specific pathogenesis of CRC has not been fully confirmed. However, it is generally believed that the risk factors for CRC include genetic and environmental components, such as genetic mutations and a

Western diet and lifestyle. CRC can develop gradually from adenomas over a long period of time. Radiation therapy and chemotherapy are generally used for the clinical treatment of CRC. Pardieck *et al.* [10] reported that immunotherapy could effectively treat CRC. Additionally, traditional Chinese medicine is a promising complementary and alternative therapy in CRC [11]. Furthermore, CRC can be prevented by a combination of drugs, diet, nutritional supplements, and exercise [12].

L1CAM is a phylogenetically conserved neural recognition molecule belonging to the immunoglobulin-like cell adhesion molecule superfamily [13]. It plays an important role in a variety of cellular processes including neurite extension, extravasation, cerebellar cell migration, and metastasis [14]. L1 is expressed in various cancers, including gliomas [15], lung cancer [16], renal carcinoma [17], melanomas [18], ovarian endometrial carcinomas [19], and

Furthermore, several reports have linked L1 expression to melanoma and prostate metastasis [17, 23-25]. For example, Thies *et al.* [24] provided evidence that L1 expression is a crucial predictor of reduced patient survival and subsequent metastasis in primary melanoma. Gavert *et al.* [25] demonstrated that the overexpression of L1 in CRC cells increases cell growth and motility and promotes liver metastasis; however, the mechanism underlying these effects remains unknown. Wachowiak *et al.* [26] detected high levels of L1CAM in the cyst fluid of glioblastomas and metastatic brain tumors. Soluble L1CAM is a cofactor for the development of tumor cysts and can promote cancer progression, making it a target for novel cancer treatments. Consistent with these previous findings, our qRT-PCR analysis indicated that *L1* transcript levels were much higher in CRC tissues than in normal intestinal tissues, providing evidence for the significant up-regulation of *L1* in CRC.

Figure 4. L1 expression detected by immunohistochemistry. A. L1 expression was detected by immunohistochemistry in poorly differentiated, well-differentiated, and lymph node metastatic adenoma tissues. The expression level of L1 is significantly higher in lymph node metastatic tissues than poorly and well-differentiated adenomas. B. ImageJ 2× was used to analyze the grayscale values obtained by immunohistochemistry. Scale-bars: 50 µm. **P<0.01.

Moos *et al.* [13] first reported the sustained activation of ERK induced by L1 under normal culture conditions and confirmed that L1 can induce both motility and invasion by an additional novel mechanism involving sustained ERK activation and motility-associated gene products. Furthermore, studies have shown that the cytoplasmic domain of L1CAM has carcinogenic activity via ERK activation [27-29]. Meier *et al.* [30] demonstrated that L1 expression in carcinomas is associated with sustained ERK activation pathway and the up-regulation of ERK-dependent motility- and invasion-associated gene products. Furthermore, Shi *et al.* [31] showed that L1 modulates cell migration and survival through the regula-

colon carcinoma [20]. L1 is highly expressed in gastrointestinal stromal tumors but not in smooth muscle tumors [21]. In colon cancer cells, the expression of L1CAM promotes tumorigenesis and metastasis. Suri *et al.* [22] injected cancer cells carrying L1CAM into mice and observed that antibodies against the ectodomain of L1CAM severely inhibited the proliferation of multiple cancer cells in culture and reduced the tumor burden. These results suggested that the L1CAM ectodomain could be an ideal target for cancer therapy.
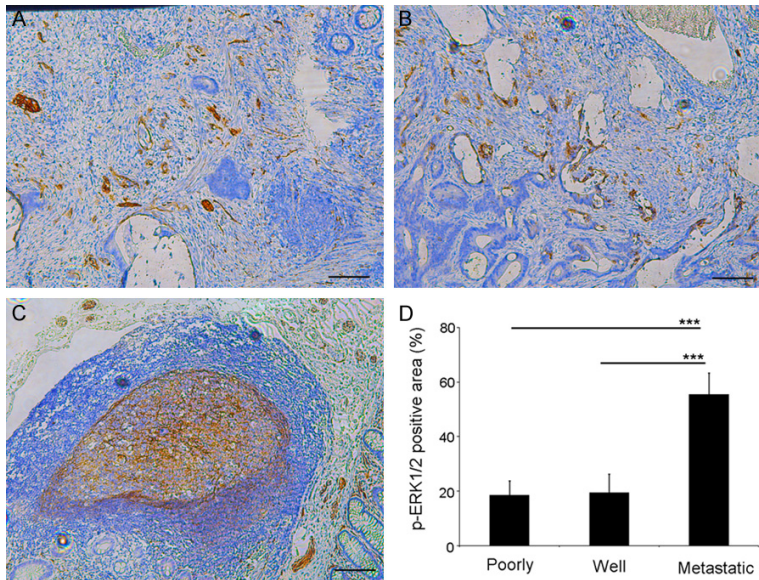
Figure 5. pERK1/2 expression detected by immunohistochemistry. pERK1/2 expression was detected by immunohistochemistry in poorly differentiated (A), well-differentiated (B), and lymph node metastatic (C) adenocarcinoma tissues. Like Ll, pERK1/2 demonstrates better activity in lymph node metastatic tissues than in poorly and well-differentiated adenomas. (D) Image J 2× was used to analyze the gray scale values obtained by immunohistochemistry. Scale-bars: 50 μm. ***$P<0.001$.
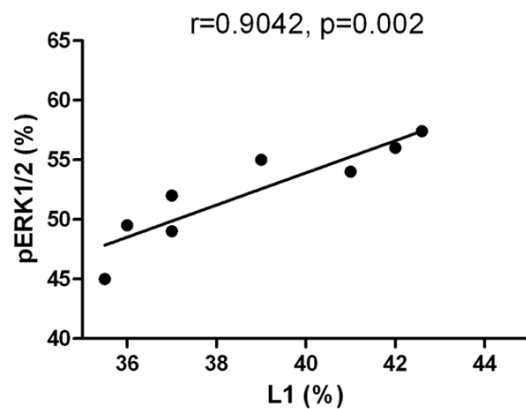

Figure 6. A linear relationship between L1 levels and pERK1/2 activity. SPSS 17.0 was used to analyze the data obtained from the L1 and pERK1/2 protein expression levels in colorectal cancer tissues. pERK1/2 is correspondingly more active when the protein expression level of L1 is higher, and the protein expression levels between them are a positively correlated linear relationship ($r=0.9042$, $P=0.002$).

tion of cell surface sialylation and fucosylation via PI3K-dependent and ERK-dependent signaling pathways. In gastric cancer, L1CAM overexpression may be a critical prognostic factor and has been significantly correlated with local

tumor cell growth, distant metastasis, and TNM stage [32]. Phospholipase D2 functions as a downstream signaling molecule of ERK to mediate the L1-dependent neurite outgrowth of cerebellar granule neurons, which may contribute to alcohol-related neurodevelopmental disorders [33]. Our results are consistent with these previous findings. Western blotting demonstrated that both L1 and pERK1/2 are highly expressed in CRC tissues, suggesting that they are probably related to the occurrence of CRC.

Most CRCs develop from adenomas, which are common lesions [1]. About 7% to 16.9% of patients with pt1 CRC have lymph node metastasis, and the survival rate in these patients is much lower than that reported in patients with pt1 cancer without lymph node metastasis [34-38]. Both quantitative and qualitative risk factors for lymph node metastasis have been reported, including poor differentiation, rectal position, tumor budding, and lymphovascular invasion [39-41]. According to previous reports [42, 43], poorly differentiated adenomas are associated with an increased risk of lymph node metastasis. Our findings demonstrate that both L1 and pERK1/2 are more highly expressed in adenomas with lymph node metastasis than in poorly differentiated and well-differentiated adenomas, indicating that L1 and pERK1/2 may be associated with the development of CRC from adenoma.

MMP13, as an ERK's downstream signaling effector, is involved in invasion and migration of breast cancer and cancer metastasis of hepatocellular carcinoma [44]. CCR4 up-regulates MMP13 expression in CRC by phosphorylating ERK1/2 [45]. Qin et al. [46] reported that PAD1 can promote tumorigenesis by regulating MEK1-ERK1/2-MMP2 signaling in human triple-negative breast cancer. Inhibition of ERK activation in human ovarian cancer cells interferes with the expression of ERK-dependent

genes integrin-β3 and cathepsin-B [47]. Previously, a study has shown that FRA1 has an essential role in the ERK signaling pathway, which was a potent regulator of cell invasion and motility in various kinds of cancer cells [48]. Consistent with these reports, our experiments also confirmed that the mRNA levels of *MMP13*, *MMP2*, *ITGB3,* and *FRA1* were highly inhibited in CRC cells pretreated with an ERK1/2 inhibitor and the protein expression of these genes was significantly higher in CRC tissues. However, whether the ERK-MMP2/MMP13/ITGB3/FRA1 signaling pathway is mediated by L1 and how L1 regulates these signaling pathways in CRC requires further validation.

In general, L1CAM and pERK1/2 are closely related to lymph node metastasis and CRC occurrence. L1 may be involved in lymph node metastasis via ERK1/2 signaling in CRC, but further studies evaluating the underlying mechanism are needed.

Disclosure of conflict of interest

None.

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Table S1. Primers used in the study

Primer ID	Primer sequence (5'-3')
L1-F	GGCACCAAATGGCTGTGAAG
L1-R	CCAAAGGCCTTCTCCTCGTT
GAPDH-F	AGAAGGCTGGGGGCTCATTTG
GAPDH-R	AGGGGCCATCCACAGTCTTC
MMP2-F	AGCGAGTGGATGCCGCCTTTAA
MMP2-R	CATTCCAGGCATCTGCGATGAG
MMP13-F	CCTTGATGCCATTACCAGTCTCC
MMP13-R	AAACAGCTCCGCATCAACCTGC
FRA1-F	GGAGGAAGGAACTGACCGACTT
FRA1-R	CTCTAGGCGCTCCTTCTGCTTC
ITGB3-F	CATGGATTCCAGCAATGTCCTCC
ITGB3-R	TTGAGGCAGGTGGCATTGAAGG

Note: L1, L1-cell adhesion molecule; MMP2, matrix metalloproteinase 2; MMP13, matrix metalloproteinase 13; FRA1, FOS-related antigen 1; ITGB3, integrin beta-3; F, forward; R, reverse.
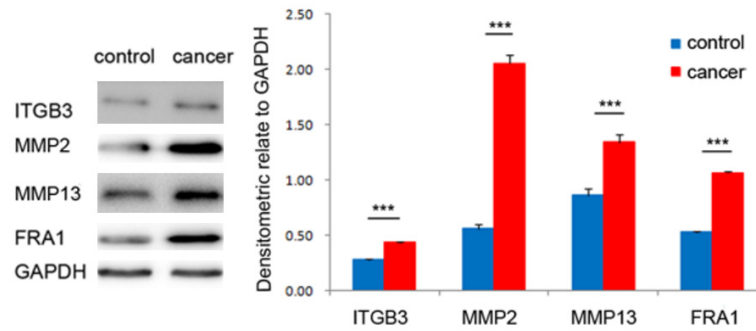

Figure S1. High protein expression of ERK1/2 activation and motility associated genes in colorectal cancer tissues. The protein expression levels of ITGB3, MMP2, MMP13, and FRA1 were detected using western blotting in normal intestinal (control) and colorectal cancer (cancer) tissues, with GAPDH adopted as the loading control. Image J 2× was used to analyze the gray scale values obtained. The levels of proteins are significantly higher, including ITGB3 (***$P<0.001$), MMP2 (***$P<0.001$), MMP13 (***$P<0.001$) and FRA1 (***$P<0.001$), in colorectal cancer than normal intestinal tissues.
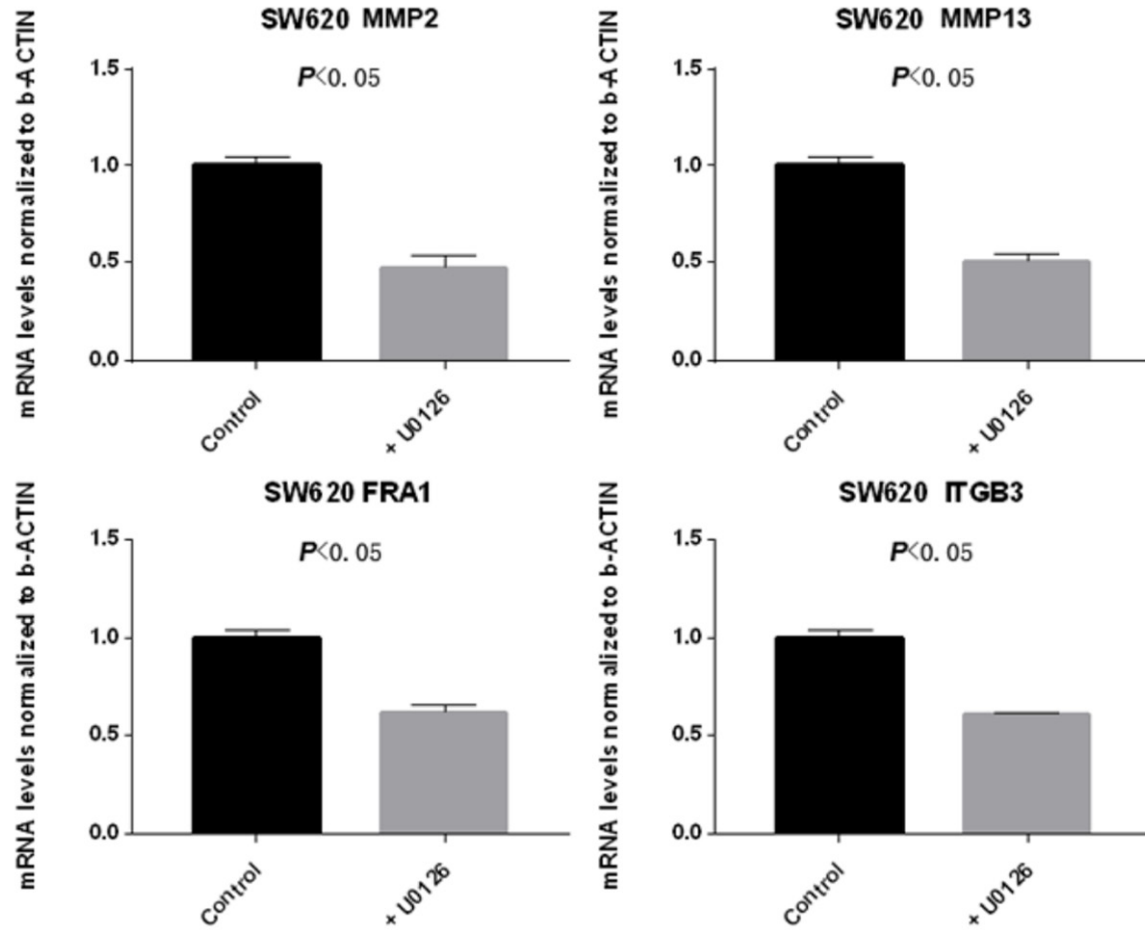
Figure S2. Inhibition of ERK1/2 expression impedes the expression of genes involved in tumor invasion and migration in colorectal cancer cells. qRT-PCR was performed to detect the mRNA levels of cancer motility-related genes (*MMP2, MMP13, FRA1, ITGB3*) in SW620 cells with ERK1/2 inhibitor (U0126). *b-ACTIN* was used as an internal reference. The mRNA levels of *MMP2* (*P*<0.05), *MMP13* (*P*<0.05), *FRA1* (*P*<0.05) and *ITGB3* (*P*<0.05) are significantly lower in SW620 cells with suppressed ERK1/2 pathway.