Am J Transl Res 2020;12(12):7797-7811
www.ajtr.org /ISSN:1943-8141/AJTR0115947

Original Article

Engulfment and cell motility 1 promotes tumor progression via the modulation of tumor cell survival in gastric cancer

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Received June 10, 2020; Accepted October 25, 2020; Epub December 15, 2020; Published December 30, 2020

Abstract: Background/aim: Engulfment and cell motility 1 (ELMO1) protein has been implicated in phagocytosis of apoptotic cells, cell migration, neurite outgrowth, cancer cell invasion and metastasis, and poor prognosis in various cancers. We investigated the role of ELMO1 in mediating the oncogenic behavior of gastric cancer (GC) cells. We also investigated the correlation between expression of ELMO1 in GC tissues and various clinicopathological parameters. Methods: We studied the impact of ELMO1 on tumor cell behavior using the pcDNA-myc vector and small interfering RNA in AGS and SNU1750 GC cell lines. We performed western blotting and immunohistochemistry to investigate the expression of ELMO1 in GC cells and tissues. Results: ELMO1 overexpression inhibited apoptosis via the modulation of PARP, caspase-3 and caspase-7 in GC cells. ELMO1 overexpression led to significant increase in the number of migrating and invading GC cells. The expression of E-cadherin decreased and that of Snail increased in GC cells upon ELMO1 overexpression. Phosphorylation of PI3K/Akt and GSK-3β was increased and that of β-catenin was decreased upon ELMO1 overexpression in GC cells. These results were reversed after ELMO1 knockdown. ELMO1 expression was significantly associated with tumor size, cancer stage, lymph node metastasis and survival. ELMO1-positive tumors had significantly higher mean of Ki-67 labeling index than ELMO1-negative tumors. There was no significant relationship between ELMO1 expression and the mean value of the apoptotic index. Conclusions: Our results indicate that ELMO1 promotes tumor progression by modulating tumor cell survival in human GC.

Keywords: ELMO1, apoptosis, prognosis, gastric cancer

Introduction

Gastric cancer (GC) is one of the major causes of cancer-related death worldwide, particularly in South Korea [1]. Despite diagnosis and treatment of GC developed, the prognosis of advanced GC is still poor with progressive behaviors-including cancer invasion and metastasis that contribute to GC-related morbidity and mortality [2, 3]. GC progression is a complex, multistep process which is regulated by alterations in a lot of tumor suppressor genes and proto-oncogenes, which are responsible for regulating cancer progression [4-6]. Therefore, to understand the pathogenesis and prognosis of GC, investigation of the molecular and biological mechanisms underlying GC progression is needed.

In humans, engulfment and cell motility (ELMO) protein family physically binds to the SH3 domain of the dedicator of cytokinesis 180 (DOCK180)-a guanine nucleotide exchange factor (GEF) of the Ras-related C3 botulinum toxin substrate (Rac) family-and enhances its GEF activity [7-10]. Consequently, the ELMO protein family plays a crucial role in Rac-dependent actin cytoskeleton rearrangement that is important in the phagocytosis of apoptotic cells, myoblast fusion, cell migration, and

dendritic spine remodeling [11-14]. The ELMO protein family is an evolutionally conserved cytoplasmic engulfment protein family with no evident catalytic activity and consists of three members, including ELMO1, ELMO2, and ELMO3 [11-14]. Previous studies have shown that ELMO1 and ELMO2 are involved in diverse cellular processes, including phagocytosis and cell migration, indicating that ELMO1 and ELMO2 have identical functions [11-14]. However, little is known about ELMO3.

Recently, it has become apparent that ELMO genes play crucial roles in pathological processes, including cancer cell invasion and metastasis [15-18]. ELMO1 is reported to be associated in metastasis of cancer cells and poor prognosis of various cancers, including leukemia, breast cancer, glioma, rhabdomyosarcoma, ovarian cancer, and hepatocellular carcinoma [19-24]. In addition, ELMO3 expression is associated with metastasis and poor prognosis of GC, colorectal cancer, non-small cell lung cancer and head and neck squamous carcinoma [25-28]. However, little is known about the function of ELMO1 in GC. In this study, we investigated whether ELMO1 affects the oncogenic behavior of GC cells and investigated its impact on prognosis of patients with GC.

Materials and methods

Cell culture and materials

GC cell lines, including SNU638, SNU1750, and SNUNCC-19, were obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea) and NCI-N87, AGS, and MKN28 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) medium supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Inc.), and 1% penicillin/streptomycin at 37°C and 5% CO_2 in a humidified atmosphere. The full-length cDNA of ELMO1 was subcloned into the pcDNA6-myc vector (Invitrogen, Thermo Fisher Scientific, Inc.). ELMO1 small interfering (si) RNA (GACAUGAUGAGCGACCUGA-dTdT) and scrambled siRNA (AccuTarget™ Negative Control siRNA) were purchased from Bioneer (Daejeon, Korea). Antibodies against cleaved poly (ADP-ribose) polymerase (PARP, cat. no. 5625), cleaved caspase-3, cleaved caspase-7, Bax, Bok, cyclin B1, p57, p27, cyclin-dependent kinase 2 (CDK2), CDK4, E-cadherin, N-cadherin, Slug, Snail, phospho-phosphatidylinositol 3-kinase/Akt (PI3K/Akt), phospho-glycogen synthase kinase (GSK)-3β, and phospho-β catenin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was obtained from Santa Cruz Biotechnology, Inc. For immunohistochemistry, primary antibodies against ELMO1 and Ki-67 were obtained from Abcam (Cambridge, UK).

Gene transfection

To overexpress ELMO1, we seeded AGS cells into 6-well plates at a density of 5×10^5 cells/well and incubated at 37°C until the cells reached 70-80% confluency. One microgram of the ELMO1-pcDNA6-myc construct (ELMO1V) was transfected using 5 μL of Lipofectamine™ 2000 reagent (Invitrogen, Thermo Fisher Scientific, Inc.). To knockdown *ELMO1*, we seeded SNU1750 cells into 6-well plates at a density of 3×10^5 cells/well and incubated at 37°C until the cells reached 40-60% confluency. ELMO1 siRNA (si-ELMO; 120 μM) was transfected using Lipofectamine™ RNAiMAX reagent (Invitrogen; Thermo Fisher Scientific, Inc.; 5 μL). Cells transfected with siRNA-negative control (siRNA-NC) and empty-pcDNA6-myc vector (EmptyV) were used as negative controls.

Cell proliferation assay

Tetrazolium salts (WST-1, Daeil Lab Inc., Seoul, South Korea) was used to assess cell proliferation. We seeded transfected cells into 96-well plates (2×10^4 cells/well) and they were maintained in a 5% CO_2 incubator at 37°C. After 2 days, 10% WST-1 reagent was added and the cells were incubated at 37°C for 1 h in a CO_2 incubator, after which the absorbance of the cells was measured at 450 nm using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Apoptosis analysis

Transfected cells were harvested and stained using 7-amino-actinomycin D and Annexin V-APC (BD Biosciences, San Jose, CA, USA) to detect apoptosis. A FACSCalibur flow cytome-

ter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and WinMDI software version 2.9 (http://winmdi.software.informer.com, The Scripps Research Institute, San Diego, CA, USA) were used to analyze the proportion of apoptotic cells.

Cell cycle analysis

Transfected cells were harvested, fixed with cold 70% ethanol, and incubated at 4°C overnight. Next, we stained the cells with propidium iodide (PI, 0.05 mg/mL) containing RNase A (0.05 mg/mL), followed by analysis of cell cycle distribution on FACSCalibur (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The proportion of the cells in the subG1, G0/G1, S, and G2/M phases was analyzed using WinMDI software version 2.9 (http://winmdi.software.informer.com, The Scripps Research Institute, San Diego, CA, USA).

Preparation of cell extracts and western blotting

Cells were washed twice with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% Triton X-100; 25 mM HEPES; 150 mM NaCl; 0.2% deoxycholate; 5 mM $MgCl_2$; 1 mM Na_3VO_4; 1 mM NaF) with Halt™ phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc.) and a protease inhibitor mixture (Complete EDTA-free protease inhibitor mixture, Thermo Fisher Scientific, Inc.). The Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Inc.) was used to normalize the protein concentration in the sample. Equivalent amounts of protein from the samples were separated electrophoretically on polyacrylamide gels that contain sodium dodecyl sulfate (SDS-PAGE) and were later transferred onto polyvinylidene fluoride (PVDF) membranes (MerkMillipore, Darmstadt, Germany). We blocked membranes with 5% BSA and then membranes were incubated with a specific primary antibody (1:1000 dilution) at 4°C overnight. On the next day, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. #7074 or #7076, Cell Signaling Technology, Inc.), and later, were developed using an enhanced chemiluminescent reagent (GE Healthcare Life Sciences, Little Chalfont, UK). Luminescent image analyzer LAS-4000 and Multi Gauge V3.0 software (Fujifilm, Tokyo, Japan) was used to analyze protein bands.

Cell invasion assay

We performed the cell invasion assay using gelatin-coated transwell chambers (Corning Incorporation, Corning, NY, USA). We seeded transfected cells onto the upper chamber of the transwell coated with 1% gelatin (Sigma, St. Louis, MO, USA). The lower chamber was filled with 0.2% BSA medium containing fibronectin (10 μg/mL, BD Biosciences, Franklin Lakes, NJ, USA). The cells that had invaded the underside of the upper chamber were stained with Hemacolor® Rapid staining solution (Merck Millipore, Darmstadt, Germany). We counted the stained cells under a light microscope in five selected fields.

Transwell migration assay

Cell migration was assessed using non-coated transwell plates (Corning Incorporation, Corning). We seeded transfected cells in the upper chamber of the transwell. In the lower chamber, 0.2% BSA containing 10% FBS was filled. The plates were then incubated for 16 h at 37°C and 5% CO_2 in a humidified incubator. Cells that had migrated to the bottom surface were fixed and stained with Hemacolor® Rapid staining solution (Merck Millipore). We counted the stained migrated cells under a light microscope in five randomly selected fields.

Patients and tissue samples

Paraffin-embedded blocks of tissue samples from 226 patients with GC that underwent surgery at the Chonnam National University Hwasun Hospital (Jeonnam, Korea) between January 2007 and December 2007 were collected. Patients with history of preoperative chemotherapy or radiation therapy before surgery were excluded. The TNM status of these specimens was evaluated according to the standardized criteria provided by the American Joint Committee on Cancer (AJCC) [29]. Clinicopathological parameters at the time of surgery were retrieved from medical records. Follow-up data were provided for all cases, for which the deadline was December 31, 2018. This study was approved by the Ethics Committee of Chonnam National University Hwasun Hospital, Jeonnam, Korea. In addition, ethical approval was obtained from the Institutional Review Board of the Chonnam National University Hwasun Hospital.

Immunohistochemistry

The paraffinized sections of GC tissues were subjected to deparaffinization and gradual rehydration. 0.01 M citrate buffer (pH 6.0, Dako, Carpentaria, CA, USA), which was heated in a pressure boiler was used to perform antigen retrieval. Endogenous peroxide activity was inhibited with Dako REAL™ peroxidase-blocking solution (Dako), and non-specific reactivity was blocked using Dako® Protein Block Serum-Free solution (Dako). The tissue sections were incubated with primary anti-ELMO1 and anti-Ki67 (diluted 1:100) antibodies overnight at 4°C in a humidified chamber. After washing with Tris-buffered saline-Tween 20 (TBS-T), the tissue sections were treated with Dako REAL™ Envision HRP/DAB detection system (Dako).

Evaluation of immunohistochemistry

Two observers blinded to the clinicopathological data of the patients assessed the immunostaining score of ELMO1 protein. The staining intensity of cancer cells was evaluated in four grades as follows: 0 (no staining), 1 (weak), 2 (medium), and 3 (strong). The extent of staining was classified into four grades based on the percentage of strongly stained area relative to that of total tumor area as follows: 0-3: 0, none; 1, <10%; 2, 10-50%; and 3, >50%. The total score was obtained by multiplying the extent and intensity of staining. Samples with a total score of ≥6 were termed ELMO1-positive samples, while those with a total score of <6 were termed ELMO1-negative. Proliferation of tumor cells was visualized using Ki67 staining. For assessing the KI, immunostained nuclei were scanned under low magnification (40 ×) and areas showing the highest concentration (hot spot) of tumor cells were selected. Hot spots were chosen from each sample, and five hot spots were examined under high magnification (400 ×). KI was defined as the number of Ki67-stained nuclei per 1000 tumor cell nuclei.

Assessment of apoptosis in patient tissues

The apoptotic index (AI) of tumor cells was assessed using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) system (Promega, Madison, MA, USA). Patient tissues were deparaffinized and rehydrated, and antigen retrieval was performed. After incubation in permeabilization solution, the terminal deoxynucleotide transferase enzyme (TdT) reaction mix to tissue sections mounted on slides was added to label patient tissues. After washing, the TdT-labeled cells were stained using the enzyme substrate 3,3-diaminobenzidine (DAB, Dako). AI was determined as the number of TUNEL positive cells per 1000 tumor cell nuclei.

Statistical analysis

Data were analyzed with the Statistical Package for Social Sciences (SPSS) software version 20.0 (IBM Corporation, Armonk, NY, USA). The association between ELMO1 expression and clinicopathological parameters was examined using Pearson's χ^2 test. We calculated survival rates by using the Kaplan-Meier method, and the significance of differences was examined using the log-rank test. The results of intergroup comparison were expressed as mean values ± SEM. Student's *t*-test was employed for comparisons between two groups. We repeated each experiment at least thrice. $P<0.05$ was considered significant.

Results

Expression of ELMO1 in various human GC cell lines

We investigated the expression of ELMO1 by western blotting in various human GC cell lines, including MKN28, AGS, SNUNCC-19, SNU638, SNU1750, and NCI-N87. Among the cell lines examined, AGS cells showed the lowest expression of ELMO1 and SNU1750 cells showed the highest expression of ELMO1 (**Figure 1A**). To control the endogenous expression of ELMO1 in AGS and SNU1750 cells, pcDNA6-myc-ELMO1 construct or ELMO1 siRNA was used respectively. The expression of ELMO1 increased significantly upon transfection with pcDNA6-myc-ELMO1 in AGS cells and it decreased upon transfection with ELMO1 siRNA in SNU1750 cells (**Figure 1B**).

Impact of ELMO1 on the proliferation of human GC cells

Cell proliferation assay was performed 2 days after the cells were transfected with pcDNA6-myc-ELMO1 or ELMO1 siRNA to elucidate the potential effects of ELMO1 expression on cell

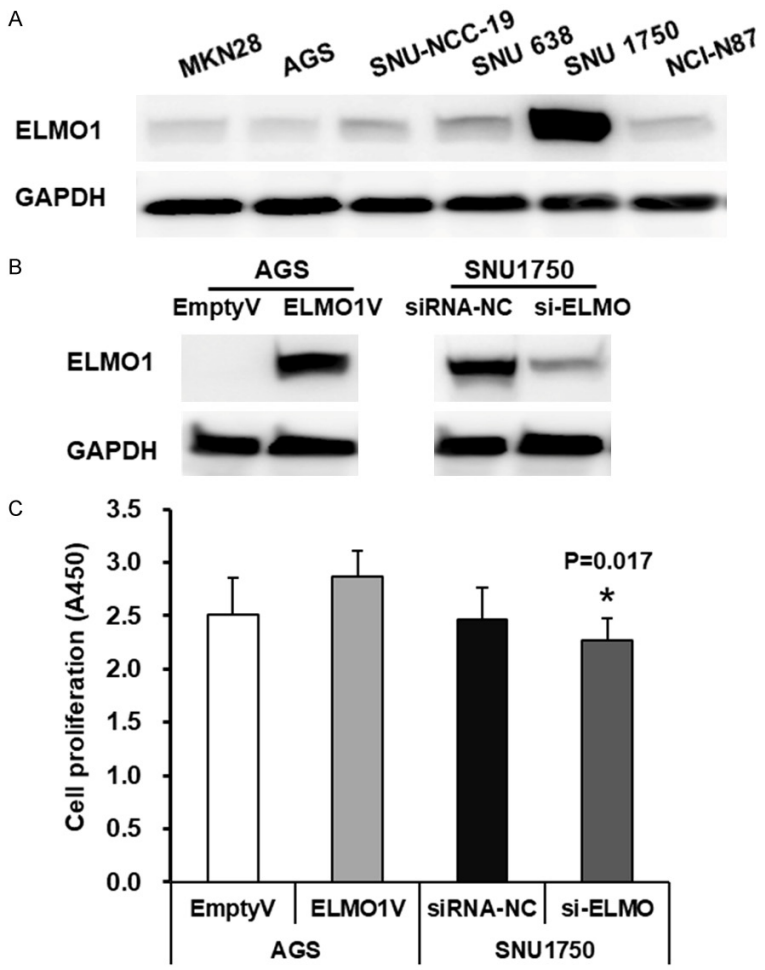


Figure 1. Expression of ELMO1 protein in human gastric cancer cells. A. Endogenous expression of ELMO1 protein in various human gastric cancer cell lines was examined by western blotting. Endogenous expression of ELMO1 protein was highest in SNU1750 cells and lowest in AGS cells. GAPDH was used as a loading control. B. ELMO1 protein was overexpressed and knocked down using ELMO1-pcDNA6-myc and ELMO1 siRNA, in AGS and SNU1750 cells, respectively. C. The impact of ELMO1 expression on the proliferation was determined by WST-1 assay in human gastric cancer cell lines. The data are representative of three independent experiments. **P*<0.05 indicates a significant difference versus the control group. ELMO1, Engulfment and cell motility protein 1; siRNA-NC, siRNA-negative control; si-ELMO, ELMO1 siRNA; EmptyV, empty-pcDNA6-myc vector; ELMO1V, ELMO1-pcDNA6-myc construct.

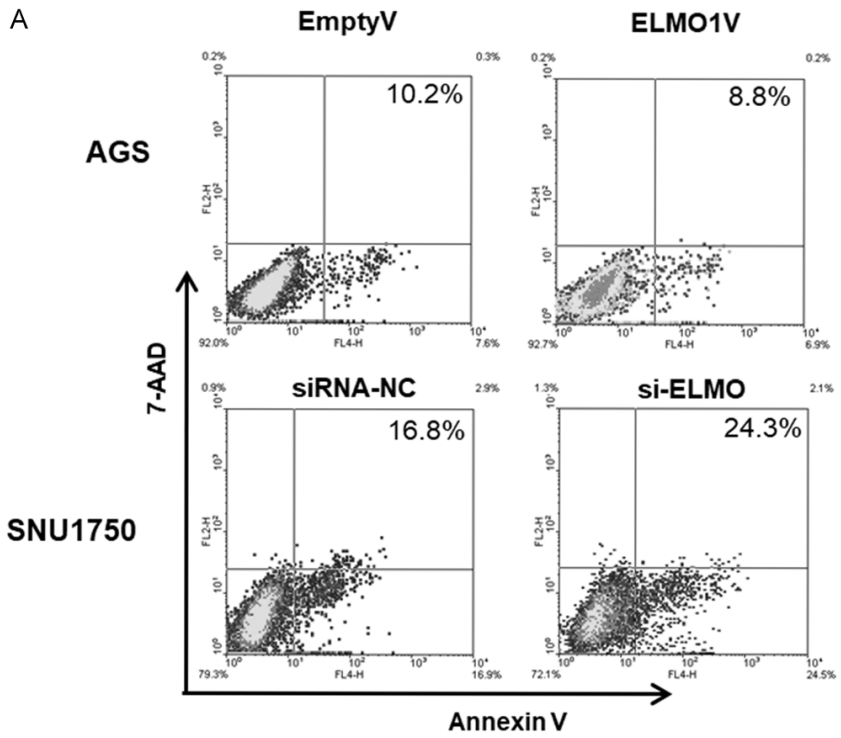
proliferation. The cell proliferation, as determined by absorbance, increased in the pcDNA6-myc-ELMO1-transfected AGS cells compared to that in the empty-pcDNA6-myc-transfected cells, although it was not statistically significant (*P* = 0.878). In contrast, cell proliferation significantly decreased in the ELMO1 siRNA-transfected SNU1750 cells, compared to that in the siRNA-negative control-transfected cells (*P* = 0.016) (**Figure 1C**).

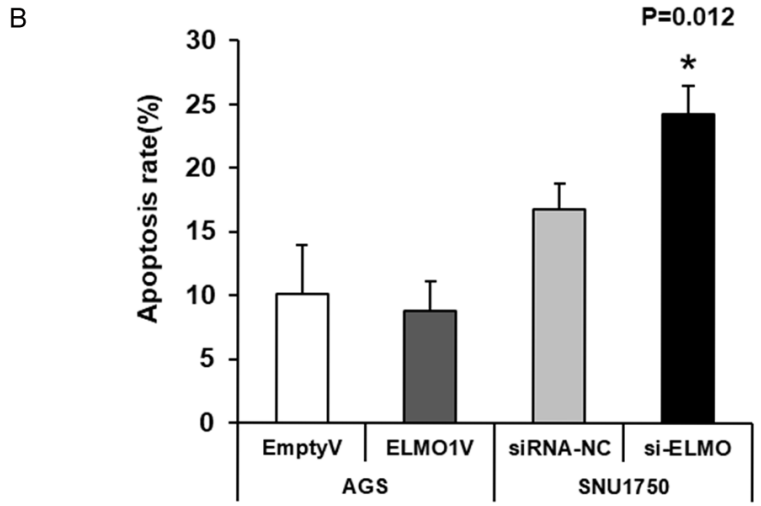
Impact of ELMO1 on apoptosis in human GC cells

We performed flow cytometric analyses to evaluate the impact of ELMO1 expression on apoptosis and cell cycle distribution. In AGS cells, the rate of apoptosis was decreased in cells transfected with the pcDNA6-myc-ELMO1 construct compared to that in cells transfected with empty-pcDNA6-myc vector (10.2 *vs.* 8.8%) (P = 0.614). The rate of apoptosis was significantly increased in SNU1750 cells after the knockdown of ELMO1 (16.8 *vs.* 24.3%) (P = 0.012) (**Figure 2A**, **2B**). To evaluate the effect of ELMO1 overexpression and knockdown on the activation of caspases, we further investigated caspase-specific activities. The expression of cleaved caspase-3, caspase-7, and PARP was downregulated in AGS cells after ELMO1 overexpression and upregulated in SNU1750 cells after ELMO1 knockdown (**Figure 2C**). We further examined whether ELMO1 expression modulates apoptosis-regulatory proteins that determines impact on apoptosis. As shown in **Figure 2C**, ELMO1 overexpression led to a decrease in the expression of the pro-apoptotic protein, Bax. In contrast, ELMO1 overexpression led to an increase in the expression of pro-apoptotic proteins, Bax and Bok.

Impact of ELMO1 on cell cycle distribution in human GC cells

ELMO1 knockdown significantly increased the proportion of cell cycle arrest in the subG1 and G0/G1 phases in SNU1750 cells (*P* = 0.028 and 0.005, respectively), while ELMO1 overexpression slightly rescued the cell cycle arrest in AGS cells (**Figure 3A**, **3B**). We investigated the effect of ELMO1 expression on posi-





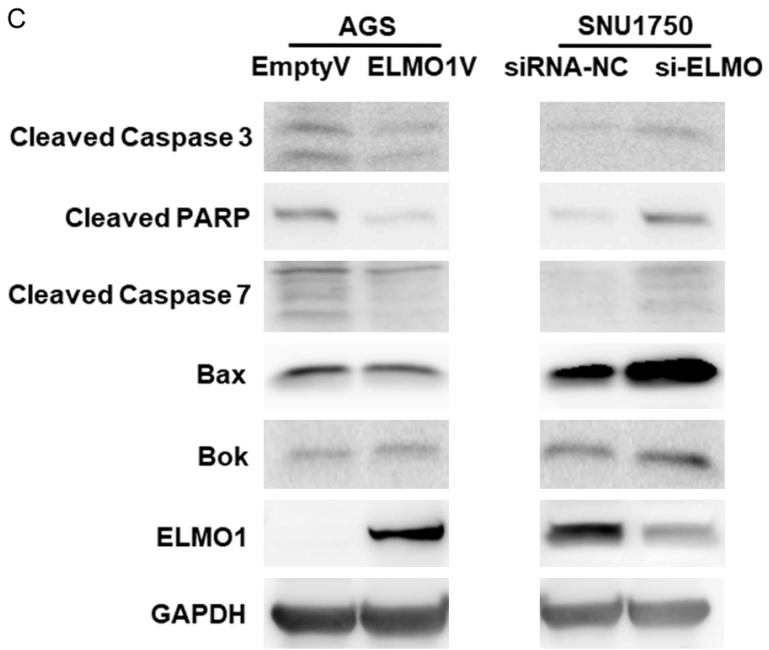


Figure 2. The impact of ELMO1 expression on apoptosis in human gastric cancer cells. A. The proportion of apoptotic cells decreased in ELMO1V-transfected AGS cells, while it increased in si-ELMO-transfected SNU1750 cells. B. The percentage of apoptotic cells was presented as the mean ± SE (n = 3; **P*<0.05). C. Expression of apoptotic and apoptosis-regulatory proteins by expression of ELMO1. Expression of cleaved caspase-3, -7 and -PARP was decreased upon ELMO1 overexpression and increased upon ELMO1 knockdown. Levels of apoptosis-regulatory proteins, Bax and Bok, were increased upon ELMO1 knockdown. Flow cytometric analyses and western blotting were performed to evaluate the impact of ELMO1 expression on apoptosis. ELMO1, engulfment and cell motility protein 1; siRNA-NC, siRNA-negative control; si-ELMO, ELMO1 siRNA; EmptyV, empty-pcDNA6-myc vector; ELMO1V, ELMO1-pcDNA6-myc construct.

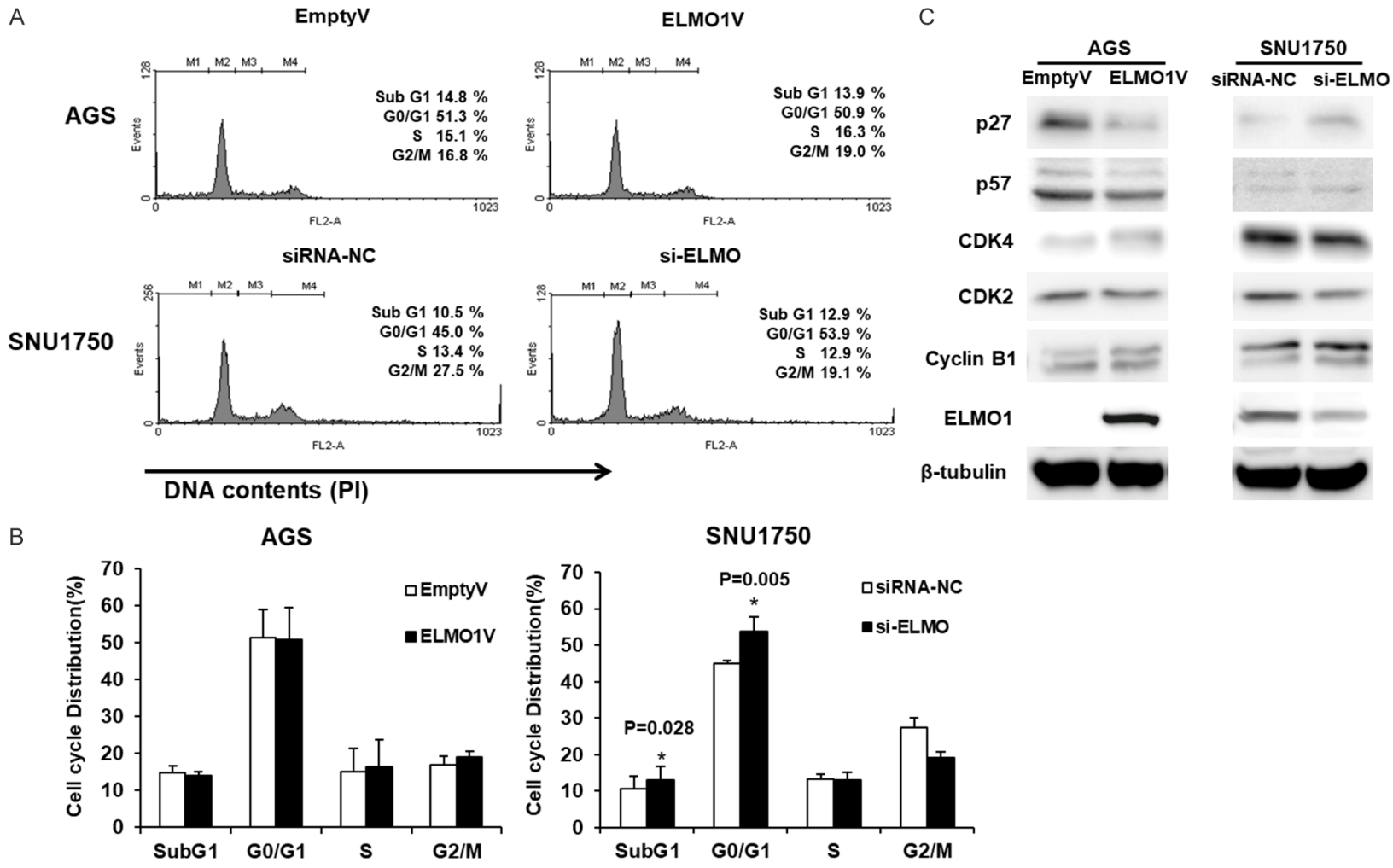


Figure 3. The impact of ELMO1 expression on cell cycle distribution in human gastric cancer cells. A. Cell cycle analysis demonstrated that ELMO1 knockdown induced cell cycle arrest in the subG1 and G0/G1 SNU1750 cells. B. The percentage of apoptotic cells was presented as the mean ± SE (n = 3; **P*<0.05). C. Expression of cyclins, CDK, and CDKI proteins by expression of ELMO1. The expression of cyclin B1 and CDK4 increased in ELMO1V-transfected cells and that of CDK2 and CDK4 protein decreased in si-ELMO-transfected cells. The protein levels of p27 and p57 were significantly decreased in ELMO1V-transfected cells and significantly increased in si-ELMO-transfected cells. Flow cytometric analyses and western blotting were performed to evaluate the impact of ELMO1 expression on cell cycle arrest. ELMO1, engulfment and cell motility protein 1; siRNA-NC, siRNA-negative control; si-ELMO, ELMO1 siRNA; EmptyV, empty-pcDNA6-myc vector; ELMO1V, ELMO1-pcDNA6-myc construct.

tive regulators such as CDKs and cyclins, and negative regulators such as CDK inhibitors (CDKIs), including p27 and p57, which are involved in cell cycle progression in human GC cells. As shown in **Figure 3C**, the expression of cyclin B1 and CDK4 significantly increased, while that of p27 and p57 significantly decreased upon ELMO1 overexpression in AGS cells. In contrast, expression of CDK2 and CDK4 significantly decreased, while that of p27 and p57 significantly increased upon ELMO1 knockdown in SNU1750 cells.

Impact of ELMO1 on the invasion and migration of human GC cells

For the invasion assay, the upper surface of the membrane in the upper chamber was coated with 1% gelatin. The number of invading pcDNA6-myc-ELMO1-transfected AGS cells was significantly increased compared to that of empty-pcDNA6-myc-transfected cells (P = 0.020). In contrast, the number of invading ELMO1 siRNA-transfected SNU1750 cells was significantly decreased relative to that of siRNA-negative control-transfected cells (P = 0.020) (**Figure 4A**). The number of migrating pcDNA6-myc-ELMO1-transfected AGS cells was increased significantly relative to that of the empty-pcDNA6-myc-transfected cells (P = 0.040). The number of migrating ELMO1 siRNA-transfected SNU1750 cells was decreased significantly relative to that of the siRNA-negative control-transfected cells (P = 0.010) (**Figure 4B**).

Impact of ELMO1 on EMT regulation in human GC cells

To investigate the phenotypic changes induced during epithelial-mesenchymal transformation (EMT) in human GC cells, the expression of well-known EMT-associated proteins, such as E-cadherin, N-cadherin, Slug, and Snail, was compared during overexpression and knockdown of ELMO1 in AGS and SNU-1750 cells. The expression of E-cadherin was decreased, while that of Snail was increased in pcDNA6-myc-ELMO1-transfected AGS cells compared to that in the empty-pcDNA6-myc-transfected cells. The expression of E-cadherin was increased, while that of Snail was decreased in ELMO1 siRNA-transfected cells compared to that in the siRNA-negative control-transfected SNU1750 cells (**Figure 5**).

Impact of ELMO1 on oncogenic signaling pathways in human GC cells

To investigate whether ELMO1 activates oncogenic signaling pathways in human GC cells, we determined the phosphorylation levels of PI3K/Akt, GSK-3β, and β-catenin using western blotting. Phosphorylation of PI3K/Akt and GSK-3β was increased, while that of β-catenin was decreased upon ELMO1 overexpression in AGS cells. In contrast, the phosphorylation of PI3K/Akt and GSK-3β was decreased, while that of β-catenin was increased upon ELMO1 knockdown in SNU1750 cells (**Figure 6**).

Correlation between ELMO1 expression and clinicopathological parameters in GC

To investigate the prognostic role of ELMO1 with respect to GC progression, we studied the expression of ELMO1 in formalin-fixed, paraffin-embedded tissue samples from 226 patients with GC using immunohistochemistry and determined the correlation between ELMO1 expression and clinicopathological parameters, including survival. In surgical specimens, the ELMO1 protein was not detected or only weakly stained in normal gastric mucosa. Immunohistochemistry of the GC specimens revealed that ELMO1 was localized in cancer cells and there was no evident expression of ELMO1 found in the stromal compartment of the samples (**Figure 7A**, **7B**). Expression of ELMO1 protein was detected in 102 of the 226 (45.1%) GC tissues that were analyzed (**Table 1**). The correlation between ELMO1 expression and clinicopathological parameters is summarized in **Table 1**. Tumor size, cancer stage, and lymph node metastasis (P = 0.019, P = 0.007, and P = 0.011, respectively) was significantly associated with ELMO1 expression. Also, the overall survival of patients with ELMO1-positive tumors was significantly lower than that of patients with ELMO1-negative tumors (P<0.001; **Figure 8**).

Correlation between ELMO1 expression and tumor cell survival in human GC

All tumor samples were subjected to the TUNEL assay and immunostaining for Ki-67 to identify tumor cell survival. The AI for the 226 tumors ranged from 0.1 to 4.4 with a mean AI of 2.1 ± 1.5. There was no significant difference between ELMO1 expression and AI (P =

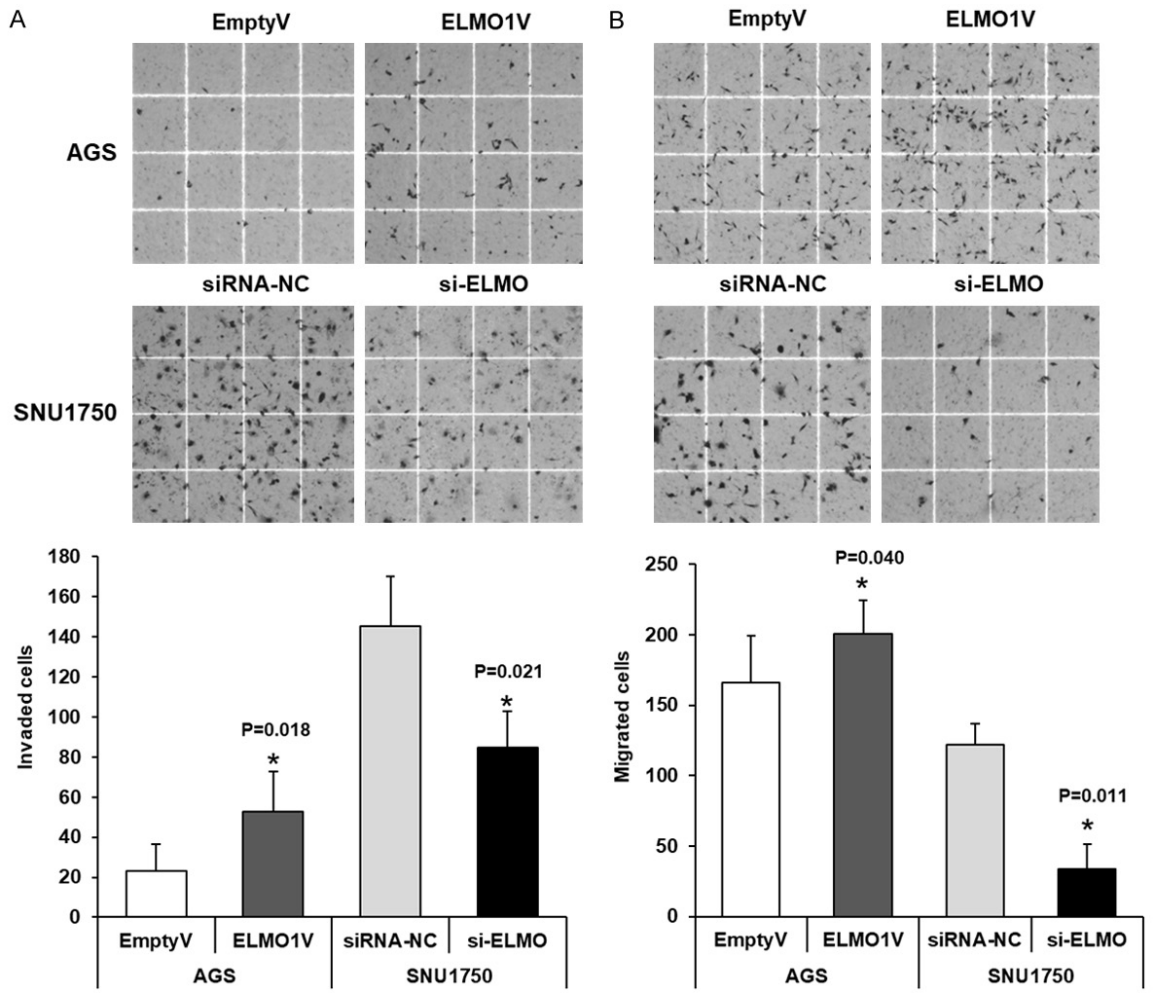


Figure 4. The impact of ELMO1 on invasion and migration of human gastric cancer cells. A. The number of invading cells was significantly increased in ELMO1V-transfected cells and significantly decreased in si-ELMO-transfected cells (mean ± SE, n = 3; **P*<0.05). Cell invasion analysis was performed using a gelatin-coated transwell chamber. B. The impact of ELMO1 on migration of gastric cancer cells. Cell migration was significantly increased in ELMO1V-transfected cells compared to that in the EmptyV-transfected cells. In contrast, cell migration was significantly reduced in si-ELMO-transfected cells compared to that in the siRNA-NC-transfected cells (mean ± SE, n = 3; **P*<0.05). The migration assay was performed using non-coated transwell chambers. ELMO1, engulfment and cell motility protein 1; siRNA-NC, siRNA-negative control; si-ELMO, ELMO1 siRNA; EmptyV, empty-pcDNA6-myc vector; ELMO1V, ELMO1-pcDNA6-myc construct.

0.520). The KI for the 226 tumors ranged from 7.1 to 64.4 with a mean KI of 24.5 ± 17.3. The mean KI value of ELMO1-positive tumors was 31.2 ± 18.6, and it was significantly higher than the KI of ELMO1-negative tumors (*P* = 0.006) (**Table 2**).

Discussion

Recently, it has been found that the ELMO1 protein family plays a key role in cell migration and cytoskeletal remodeling via the activation of RAC1, and it is associated with metastasis and poor prognosis in various cancer types [15-24].

GC is caused due to the accumulation of activating mutations in proto-oncogenes and inactivating mutations in tumor suppressor genes that regulate tumor cell growth [4-6]. Previously, ELMO1 overexpression was associated with increased cell growth, invasion, migration, and poor prognosis in various human cancer types [19-24]. In this study, ELMO1 overexpression enhanced the invasion and migration ability of human GC cells. In contrast, ELMO1 knock-

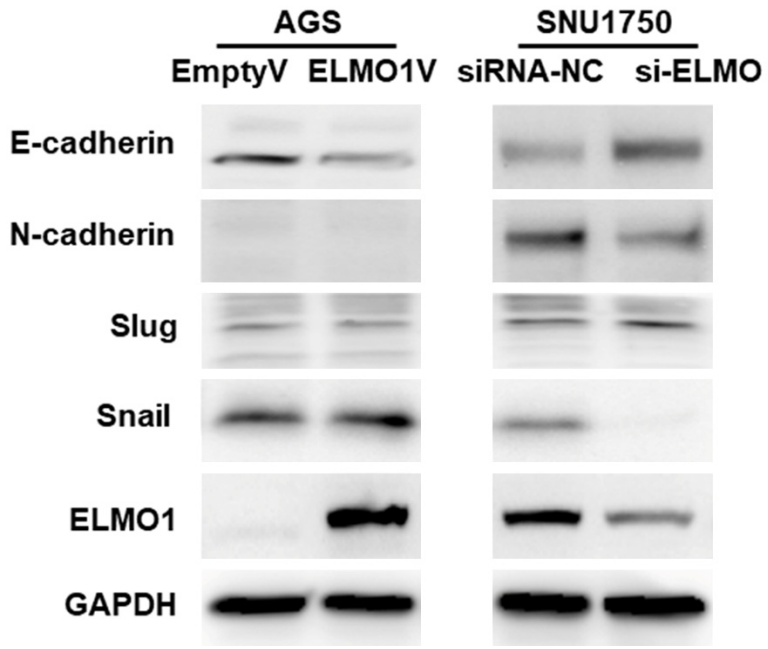


Figure 5. The impact of ELMO1 on EMT modulators in human gastric cancer cells. The expression of E-cadherin was downregulated, while that of Snail was upregulated in ELMO1V-transfected cells. In contrast, the expression of E-cadherin was increased, while that of Snail was significantly decreased in si-ELMO-transfected cells. Western blotting was performed to evaluate the impact of ELMO1 expression on EMT. ELMO1, engulfment and cell motility protein 1; EMT, epithelial to mesenchymal transition; siRNA-NC, siRNA-negative control; si-ELMO, ELMO1 siRNA; EmptyV, empty-pcDNA6-myc vector; ELMO1V, ELMO1-pcDNA6-myc construct.

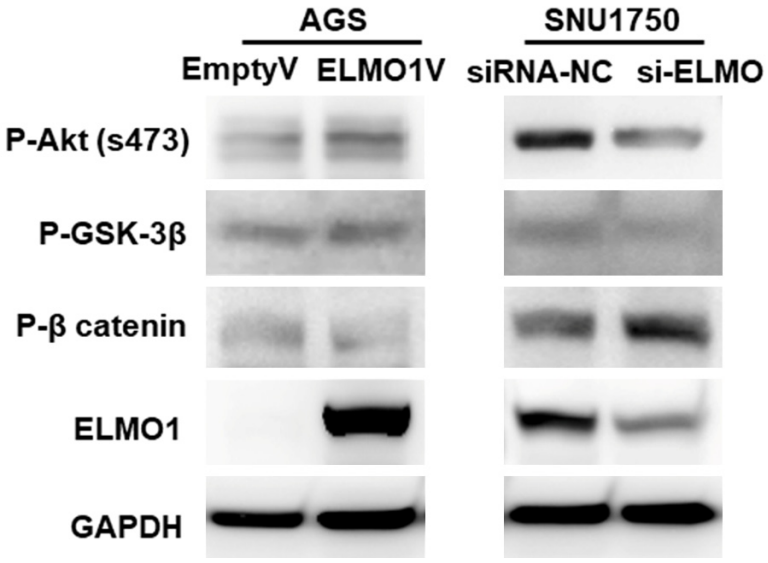


Figure 6. The impact of ELMO1 on intracellular signaling pathways in human gastric cancer cells. PI3K/Akt and GSK-3β phosphorylation was increased, while β-catenin phosphorylation was decreased in ELMO1V-transfected cells. In contrast, PI3K/Akt and GSK-3β phosphorylation was decreased, while β-catenin phosphorylation was increased upon ELMO1 knockdown. Western blotting was performed to evaluate the impact of ELMO1 expression on intracellular signaling pathways. ELMO1, engulfment and cell motility protein 1; siRNA-NC, siRNA-negative control; si-ELMO, ELMO1 siRNA; EmptyV, empty-pcDNA6-myc vector; ELMO1V, ELMO1-pcDNA6-myc vector; PI3K/Akt, phosphatidylinositol 3-kinase/Akt; GSK-3β, glycogen synthase kinase-3β.

down inhibited tumor cell invasion and migration. These results indicate that ELMO1 plays an oncogenic role in GC progression and metastasis.

Apoptosis or programmed cell death regulates many biological events, including cell proliferation, cell death, and tissue homeostasis [30, 31]. Defects in the regulation of apoptosis contribute to the pathogenesis of human diseases. Evasion of apoptosis is one of the important hallmarks of many cancers, and results in the ability to sustain unscheduled proliferation [32, 33]. In our study, we confirmed that transient overexpression of ELMO1 promotes migration and invasion of cancer cells which is necessary for colon cancer. ELMO1 knockdown significantly increased cell apoptosis, via the modulation of caspase-3, caspase-7, and PARP in human GC cells. In addition, a previous study showed that ELMO1 overexpression protects endothelial cells from apoptosis via reduced caspase-3 and caspase-7 activities [34]. However, in our study, ELMO1 overexpression did not significantly inhibited apoptosis. This suggests that it may be difficult to expect dramatic inhibition of apoptosis by transient overexpression of ELMO1 in cancer cells that have already suppressed apoptosis.

The cell cycle constitutes of a many thoroughly integrated

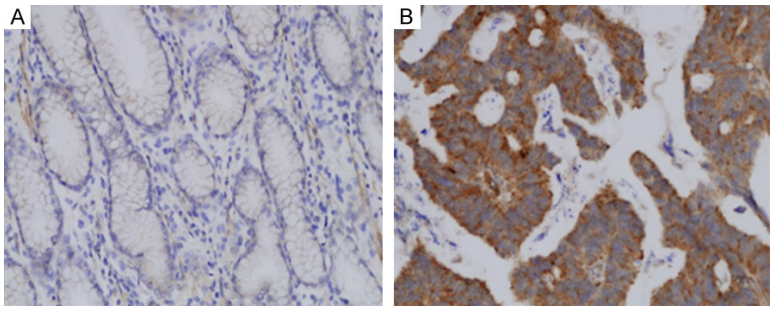


Figure 7. The expression of ELMO1 in human gastric cancer tissues. Gastric tissue samples were stained with antibodies against ELMO1 by immunohistochemistry. A. ELMO1 expression in normal gastric mucosa (× 200). ELMO1 protein remained unstained or was only weakly stained in normal gastric mucosa. B. ELMO1 expression in gastric cancer tissues (× 200). ELMO1 expression was predominantly observed in the cytoplasm of gastric cancer tissues. ELMO1, engulfment and cell motility protein 1.

Table 1. Association between ELMO1 expression and clinicopathological parameters in human gastric cancer

	Total (n = 226)	ELMO1 expression		P-value
		Negative (n = 124)	Positive (n = 102)	
Age (years)				0.157
≥58.5	89	54	35	
<58.5	137	70	67	
Sex				0.180
Male	149	77	72	
Female	77	47	30	
Tumor size (cm)				0.019
≥4.8	130	80	50	
<4.8	96	44	52	
Stage				0.007
I/II	139	86	53	
III/IV	87	38	49	
Histological type				0.706
Well-differentiated	56	34	22	
Moderately differentiated	33	16	17	
Poorly differentiated	99	54	45	
Signet	38	20	18	
Depth of invasion (T)				0.065
T1/T2	126	76	50	
T3/T4	100	48	52	
Lymph node metastasis (N)				0.011
N0	123	77	46	
N1-3	103	47	56	

ELMO1, engulfment and cell motility protein 1.

events that allow the cell to grow and proliferate. This process is critical to tissue homeostasis and development. Cell cycle is a complex process driven by several positive and negative regulators, including cyclins, CDK, and CDK inhibitors [35, 36]. The dysregulation of cell cycle may lead to uncontrolled cell proliferation similar to that is observed in cancer. Cancer-related cell cycle dysregulation is often mediated via alterations in the activity of diverse cell cycle regulators [37, 38]. In this study, the levels of positive regulators of cell cycle progression, including CKD4 and cyclin B1, were significantly increased, while those of negative regulators, including CDKIs, p27 and p57, were significantly decreased upon ELMO1 overexpression in GC cells. These results were reversed after ELMO1 was knocked down. Therefore, ELMO1 may attribute to GC progression through cell cycle dysregulation.

EMT is one of the key processes involved in carcinogenesis and progression, and is characterized by upregulation of mesenchymal markers, such as Snail and vimentin and downregulation of epithelial markers, such as E-cadherin [39-41]. Previous study that used liver cancer cell lines and liver tumor xenograft model demonstrated that ELMO1 induces EMT *in vitro* and *in vivo* [42]. Therefore, we investigated the expression of EMT-associated proteins in human GC cells by western blotting. The expression of E-cadherin was decreased and that of Snail was increased upon ELMO1 overexpression. These results were reversed upon ELMO1 knockdown. This indicates the positive relationship between ELMO1 expression and EMT induction in human GC cells.

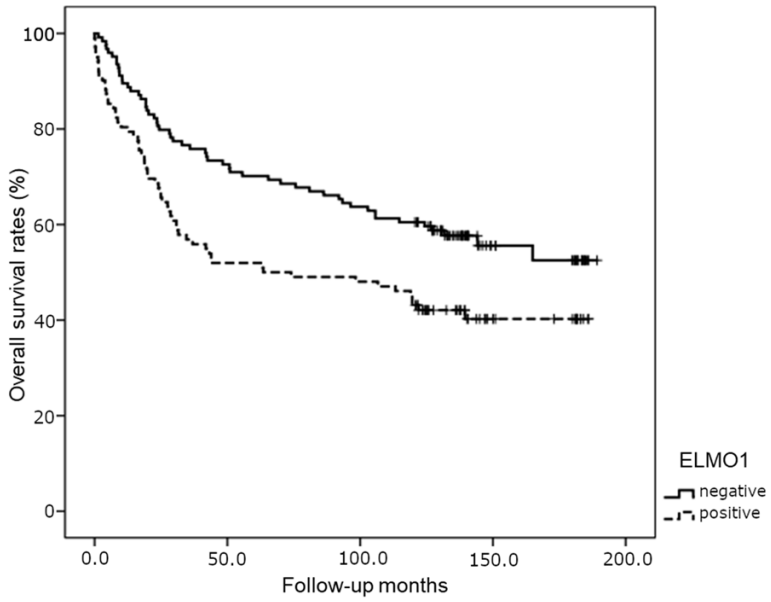


Figure 8. Overall survival rates of patients with gastric cancer with positive (solid line) and negative (dotted line) expression of ELMO1. Overall survival estimates were analyzed by the Kaplan-Meier method analysis with log-rank test. Positive expression of ELMO1 was associated with poor survival in patients with gastric cancer (P<0.001). ELMO1, engulfment and cell motility protein 1.

Table 2. Association between ELMO1 expression and tumor cell survival in human gastric cancer

Parameters (Mean ± SD)	Total (n = 226)	ELMO1 expression		P-value
		Negative (n = 124)	Positive (n = 102)	
KI	24.5 ± 17.3	18.2 ± 13.7	31.2 ± 18.6	0.006
AI	2.1 ± 1.5	2.1 ± 1.5	2.1 ± 1.8	0.520

ELMO1, engulfment and cell motility protein 1; SD, standard deviation; KI, Ki-67 labeling index; AI, apoptotic index.

PI3K/Akt, GSK-3β, and β-catenin signaling pathways play a pivotal role in numerous cellular processes, including cell apoptosis, DNA repair, cell proliferation, cell cycle, angiogenesis, signaling, and metabolic pathways [43-45]. In addition, the regulatory mechanisms and biological functions of PI3K/Akt, GSK-3β, and β-catenin signaling are implicated in many human disorders including inflammation, neurodegenerative diseases, and cancers [43-45]. The molecular mechanism linking ELMO1 expression with alteration of oncogenic phenotypes in human GC cells is unclear. Previously, genes encoding for proteins involved in the PI3K/Akt signaling pathway were found to be significantly overexpressed in ELMO1-overexpressed liver tumors. Importantly, the levels of phosphorylated PI3K/Akt and GSK-3β were increased upon ELMO1 overexpression, whereas the levels were decreased upon downregulation of ELMO1 in liver cancer cells [42]. We measured the phosphorylation level of PI3K/Akt, GSK-3β, and β-catenin using western blotting to investigate the contribution of specific intracellular signaling pathways that are involved in the regulation of ELMO1 expression in human GC cells. The phosphorylation of PI3K/Akt and GSK-3β was increased and that of β-catenin was decreased upon ELMO1 overexpression in human GC cells. In contrast, the phosphorylation of PI3K/Akt and GSK-3β was decreased and that of β-catenin was increased upon ELMO1 knockdown in human GC cells. These results indicate that ELMO1 might regulate GC cell behavior via activation of PI3K/Akt, GSK-3β, and β-catenin signaling.

ELMO1 expression is increased in numerous human cancer types and has been linked with cancer development and progression [19-24]. In our study, ELMO1 expression in GC tissues was upregulated compared to that in normal mucosa. Next, we assessed the expression of ELMO1 and its prognostic value in human GCs with clinicopathological data, including survival. ELMO1 expression was significantly associated with tumor size, cancer stage, lymph node metastasis, and survival. These results suggest that ELMO1 may play an important role in gastric carcinogenesis and may be useful as a prognostic marker in patients with GC.

Finally, we investigated the relationship between ELMO1 expression, and apoptosis and proliferation in human GC tissues to confirm the results obtained from studies on human GC cell lines. We found that the mean KI and MVD values of ELMO1-positive tumors were

significantly higher than those of ELMO1-negative tumors. However, there was no significant difference between ELMO1 expression and AI. These results confirmed the proliferation promoting potential of ELMO1 *in vivo*; this was consistent with the results of *in vitro* studies.

Taken together, the results of this study indicate that ELMO1 promotes tumor progression via modulating tumor cell proliferation in human GC.

Acknowledgements

This study was supported by a grant (BRCI-18008) of Chonnam National University Hospital Biomedical Research Institute, Republic of Korea, and partly by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (NRF-2017R1A2B-4004703 and NRF-2019R1F1A1063463).

Disclosure of conflict of interest

None.

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