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Research Paper

Cell Recognition Molecule L1 Regulates Cell Surface Glycosylation to Modulate Cell Survival and Migration

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Abstract

***Background*:** Cell recognition molecule L1 (L1) plays an important role in cancer cell differentiation, proliferation, migration and survival, but its mechanism remains unclear.

***Methodology/Principal*:** Our previous study has demonstrated that L1 enhanced cell survival and migration in neural cells by regulating cell surface glycosylation. In the present study, we show that L1 affected cell migration and survival in CHO (Chinese hamster ovary) cell line by modulation of sialylation and fucosylation at the cell surface via the PI3K (phosphoinositide 3-kinase) and Erk (extracellularsignal-regulated kinase) signaling pathways. Flow cytometry analysis indicated that L1 modulated cell surface sialylation and fucosylation in CHO cells. Activated L1 upregulated the protein expressions of ST6Gal1 (β-galactoside α-2,6-sialyltransferase 1) and FUT9 (Fucosyltransferase 9) in CHO cells. Furthermore, activated L1 promoted CHO cells migration and survival as shown by transwell assay and MTT assay. Inhibitors of sialylation and fucosylation blocked L1-induced cell migration and survival, while decreasing FUT9 and ST6Gal1 expressions via the PI3K-dependent and Erk-dependent signaling pathways.

***Conclusion*:** L1 modulated cell migration and survival by regulation of cell surface sialylation and fucosylation via the PI3K-dependent and Erk-dependent signaling pathways.

Key words: Cell adhesion molecule L1; Glycosylation; Sialylation; Fucosylation; CHO cells.

Introduction

Metastatic cancer cells usually express high density of sialic acid-rich glycoproteins on cell surfaces and help cancer cells enter the circulatory system [1]. Glycosylation is a post- or co-translational modification for most proteins and play important roles in cancer development [2]. In a previous study, we have demonstrated that the upregulation of cell adhesion molecule L1 (L1) in neural cells increased the expressions of sialic acid and fucose on the cell surface, which subsequently, enhanced cell survival [3]. Fucosylation is a common modification involving oligosaccharides and many synthesis pathways are involved in the regulation of fucosylation [4, 5]. Fucosylation of glycoproteins modulates the biological functions of adhesion molecules and plays an important role in cell survival and metastasis [6].

L1 is a type of transmembrane cell adhesion glycoprotein which belongs to a large immunoglobulin superfamily of cell adhesion molecules and mediates interactions between cells [7]. L1 promotes cell survival, migration and axon guidance in the nervous system [8]. The overexpression of L1 has been shown to indicate poor prognosis in a variety of human carcinomas including ovarian, lung, gastric, colorectal and pancreatic cancers [9-13]. Recently, we have demonstrated that

L1 upregulated the protein expressions of ST3Gal4 and FUT9 via activation of the PLCγ (Phospholipase Cγ) pathway, which increased cell surface sialylation and fucosylation [14]. CHO cell line was derived from the Chinese hamster ovary and can provide a high expression of recombinant glycoproteins which are equipped with a glycosylation mechanism very similar to that found in humans [15]. Sialic acid occupies the terminal end on oligosaccharide chains in these glycoproteins and influences the biological behavior of cells [16]. Previous studies have demonstrated that L1 regulated the Erk signaling pathway [17]. Cells expressing L1 activated the phosphoinositide 3-kinase/ Protein kinase B (PI3K/Akt) pathway to stimulate motility in gastric cancer and induce proliferation in renal cell carcinoma [18]. However, the precise mechanism of L1 in cell migration and survival is still unclear.

In this study, we investigated the effects of L1 on CHO cell survival and migration by regulation of cell surface glycosylation. We demonstrate that L1 regulated cell surface sialylation and fucosylation via the PI3K and Erk signaling pathways.

Results

L1 modulated the expression of specific carbohydrates on the cell surface of CHO cell line

Given that L1 is one of many carbohydrate-carrying molecules at the cell surface and mediates interactions between other adhesion molecules in the nervous system, we hypothesized that L1 might modulate specific glycosylation patterns at cell surfaces. To test this hypothesis, we compared cell surface glycosylation patterns between CHO cells and L1-transfected CHO (L1-CHO) cells by flow cytometry. The expression of carbohydrates recognized by SNA (Sambucus nigra lectin) and L5 antibodies were significantly upregulated in L1-transfected versus non-transfected CHO cells (**Fig. 1**). SNA recognized terminal sialic acids while L5 antibodies recognized terminal fucose (**Fig.2A**). These results demonstrated that L1 plays a role in modulation of the sialylation and fucosylation at cell surfaces.

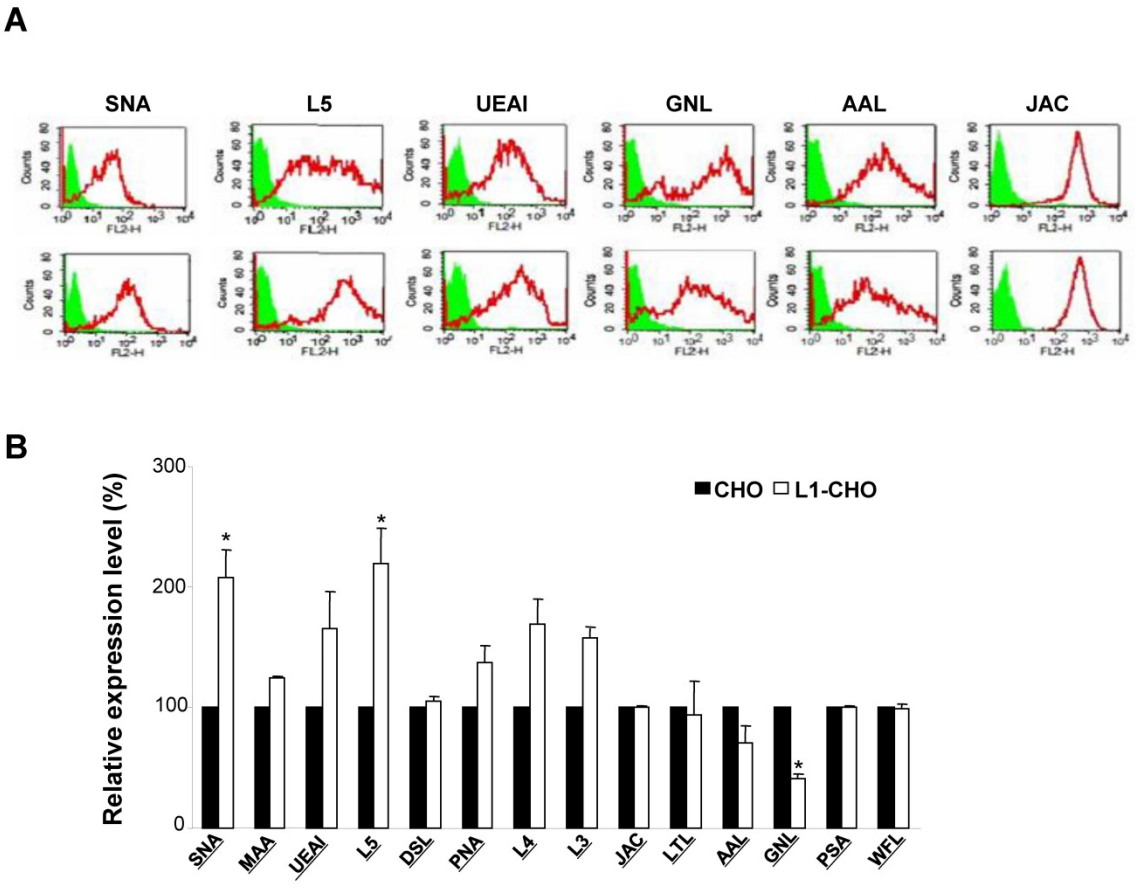

Figure 1. Glycosylation patterns on cell surface of CHO cells and L1-transfected CHO cells. CHO cells and L1-CHO cells were subjected to flow cytometry analysis using a panel of carbohydrate surface markers, including lectins and antibodies against carbohydrates. **A.** In the flow cytometry histograms, the areas in green show the number of unstained cells and the areas outlined in red represent cells binding to carbohydrates antibodies L5 and various lectins including SNA (Sambucus nigra lectin), MAA (Maackia amurensis lectin), UEAI (Ulex europaeus agglutinin I), DSL (Datura stramonium lectin) and JAC (Jacalin). **B.** The quantitative results showed that the expression of carbohydrates recognized by SNA as well as L5 antibodies were significantly increased in L1-CHO cells versus CHO cells. *: $p<0.05$, by Student's test.

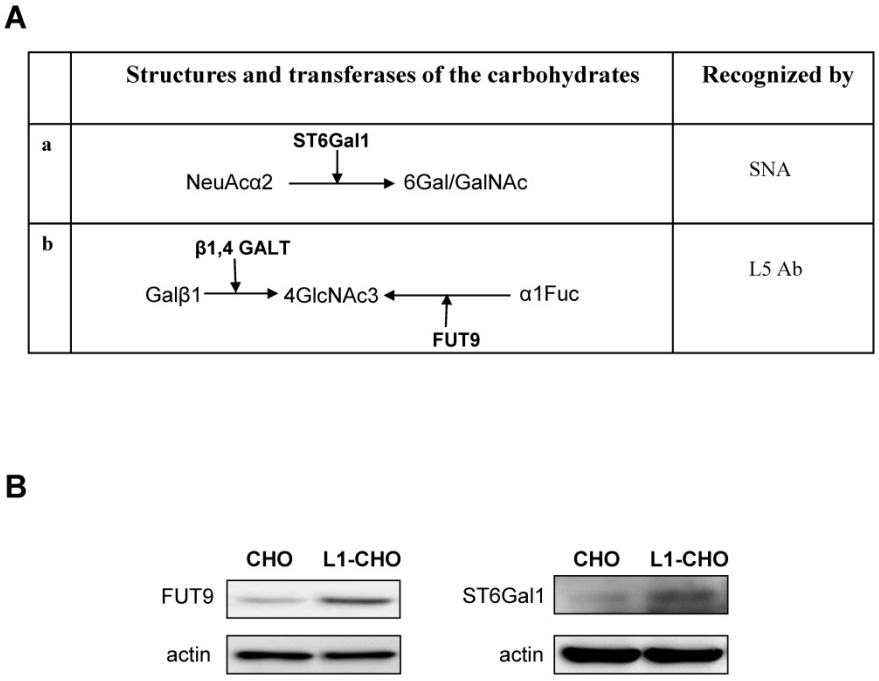

Figure 2.The protein expressions of ST6Gal1 and FUT9 were modulated by L1. A. The carbohydrate structures for terminal sialylation (a and b) and fucosylation (b) with related transferases were recognized by SNA and L5 antibodies on cell surfaces. **B.** Western blot was used to detect the expression of transferases. The protein expressions of ST6Gal1 and FUT9 were significantly upregulated in the L1-CHO cells versus CHO cells.

L1 regulated the expression of sialyltransferases, ST6Gal1 and fucosyltransferase, FUT9

Since L1 is involved in the regulation of sialylation and fucosylation at cell surfaces, we hypothesized that activated L1 may regulate the expression of specific sialyltransferases and fucosyltransferases. Western blot was used to assess this hypothesis. The results showed that the expressions of FUT9 and ST6Gal1 were significantly upregulated in CHO cells transfected with L1 versus non-transfected CHO cells (**Fig.2B**). Hence, the protein expressions of ST6Gal1 and FUT9 in CHO cells were upregulated upon L1 activation, indicating changes in sialylation and fucosylation activities.

Activated L1 promoted cell migration of CHO cells

To investigate the role of activated L1 in cell migration, transwell membranes were coated with L1 antibodies (L1Ab). Thus, only cells that express L1 at the cell surface will be stimulated. As expected, under such conditions, cell migration was significantly increased in L1-CHO cells treated with L1Ab, compared to L1Ab-treated non-transfected CHO cells (**Fig. 3A, 3B**).

Activated L1 promoted cell survival of CHO cells

To investigate whether L1 plays a role in cell survival, MTT analysis was performed. In agreement with our previous study, cell survival was significantly enhanced in L1-CHO cells versus CHO cells (**Fig. 3C**). Together, these observations demonstrated that changes in glycosylation patterns induced by L1 may also regulate cell migration and cell survival.

Inhibitors of sialylation and fucosylation blocked L1-induced cell migration and cell survival

We investigated whether sialylation and fucosylation could be involved in L1-inducedcell migration and survival by using Soyasaponin I, a potent and specific sialyltransferase inhibitor, and Tunicamycin, which prevents N-glycosylation of fucosyltransferase leading to inactivation of the enzyme. Both Tunicamycin and Soyasaponin I could significantly decreased the cell migration of L1-CHO cells after L1 antibody stimulation in a dose-dependent manner (**Fig 4A**). Additionally, cell survival of L1-CHO cells stimulated with L1 antibody were significantly reduced after treatment with Soyasaponin I and Tunicamycin in a dose-dependent manner (**Fig 4B**). The strongest inhibition effects were produced after the sialyltransferase inhibitor and fucosyltransferase inhibitor were used together (**Fig 4C and 4D**). The results demonstrated that sialylation and fucosylation may also contribute to L1-induced cell migration and cell survival.

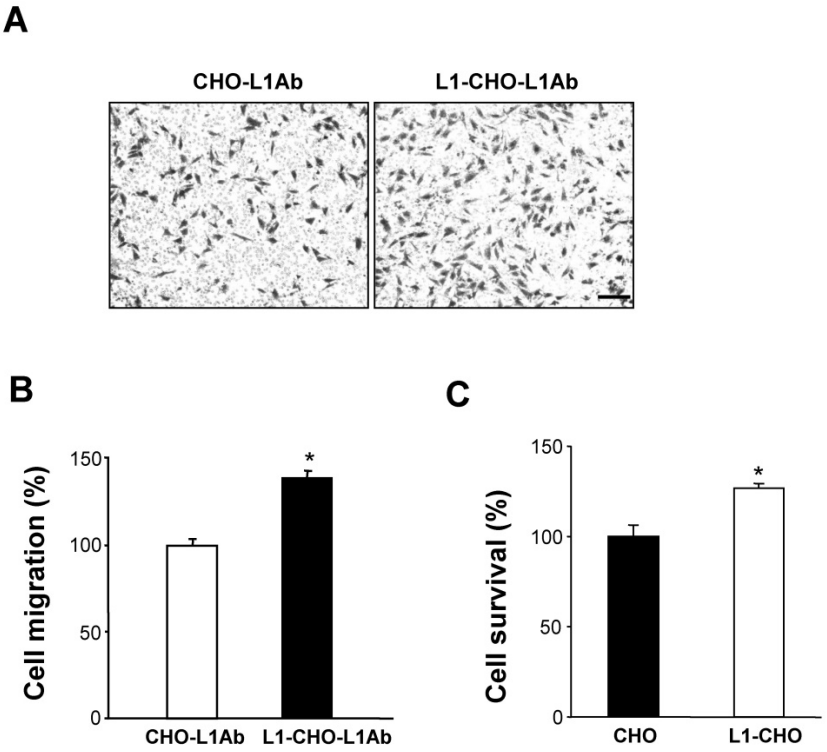

Figure 3. Activation of L1 promoted cell migration and cell survival. To verify the role of L1 in cell migration, the undersurface of transwell membranes was coated with L1Ab. Relative number of cells transmigrating through the membrane were determined by staining cells on the undersurface of the transwell membrane followed by cell lysis and measurement of absorbance value. Optical density measurements of the dye were directly proportional to numbers of migrated cells. **A.** Cell migration was significantly enhanced in L1-CHO cells after treatment with L1Ab (L1-CHO-L1Ab) versus L1Ab-treated non-transfected CHO cells (CHO-L1Ab). Photomicrographs illustrate the greater density of migrated L1-CHO cells treated with L1Ab (L1-CHO-L1Ab) on the undersurface of the membrane. **B.** Quantitative measurements from Figure 3A. **C.** To investigate the role of L1 in cell survival, MTT analysis was performed. Cell survival was significantly enhanced in the L1-CHO cells versus CHO cells. *: $p<0.05$, by Student's test.

L1 induced the upregulation of FUT9 and ST6Gal1 protein expressions, while L1-induced cell migration and survival are dependent on thePI3K and Erk signaling pathways

To understand the mechanism of L1 in modulating sialylation and fucosylation, we investigated the protein expressions of FUT9 and ST6Gal1 after inhibition of signal transduction pathways known to be activated by L1. In L1Ab-treated L1-CHO cells, both PI3K inhibitor (LY294002) and Erk inhibitor attenuated cell migration (**Fig. 5A**). PLCγ inhibitor, U73122, PI3K inhibitor, JNK inhibitor, caffoline and Erk inhibitor reduced cell survival in L1-CHO cells, whereas Cdc25 phosphatase inhibitor II and protein kinase A inhibitor (KT5720) had no effect on cell survival and migration (**Fig. 5B**). In addition, PI3K inhibitor and Erk inhibitor reduced both FUT9 and ST6Gal1 protein expressions (**Fig. 5C**). The down-regulation of FUT9 and ST6Gal1 protein expressions together with the reduction of L1-induced cell migration and survival were observed only when cells were treated with PI3K inhibitor and Erk inhibitor. Together, the data suggest that L1 regulates sialylation and fucosylation to modulate cell function via PI3K-dependent and Erk-dependent pathways.

Discussion

L1, a member of the cell adhesion molecule superfamily, consists of five fibronectin type III repeats, six immunoglobulin-like domains, a single transmembrane region and a short cytoplasmic tail [19, 20]. The protein, encoded by the L1 gene, is a type of glycoprotein belonging to the immunoglobulin superfamily. L1 is able to bind to a number of other proteins or "homophilically" to itself [21]. Complex N-glycans are distinct and may regulate surface glycoprotein levels, cell differentiation and cell proliferation [22]. L1 plays an important role in the development of the nervous system by glycosylation [23]. Overexpression of L1 has been reported in a variety of cancers, including thyroid carcinoma, non-small cell lung cancer, gastric cancer, hepatocellular carcinoma, pancreatic cancer, colorectal cancer, ovarian cancer and endometrial cancer [24-27]. These findings implied that L1 also plays a role in human carcinogenesis. However, the mechanism in which L1 modulates cell migration, proliferation, invasion and apoptosis remains unknown [28, 29]. Previously, we have demonstrated that L1 interacted with several carbohydrates on cell surfaces and modulates cell function. In the present

study, we show that L1 utilized the same mechanism together with the PI3K and Erk signal transduction systems to regulate cell survival and migration.

CHO cells produce recombinant glycoproteins via a glycosylation machinery which is similar to human cells. In this study, we have confirmed the expression of carbohydrates recognized with SNA or L5 antibody upon upregulation of L1 expression in CHO cells. SNA or L5 antibody can recognize terminal sialic acids or fucose, respectively. *N*-acetylglucosamine (GlcNAc) contributes to the function and structure of cells and is the precursor of *N*-acetylgalactosamine (GalNAc). GlcNAc is converted to sialic acid which is the terminal glycan in many glycosylated proteins. We have shown that ST6Gal1 was recognized by SNA and NeuAcα2 converted to GalNAc was increased in L1-CHO cells. Similarly, FUT9 was recognized by L5 antibody and α1Fuc converted to 4GlaNAc3 was augmentated in L1-CHO cells. This indicated that L1 may play a central role in modulation of sialylation and fucosylation by increasing the expression of ST6Gal1 and FUT9 on cell surfaces. Furthermore, we found that cell migration was significantly increased in L1-CHO cells treated with L1Ab, but not in L1Ab-treated CHO cells. Cell survival was also significantly enhanced in L1-CHO cells versus non-transfected CHO cells. Moreover, L1-induced cell migration and survival were repressed when sialylation and fucosylation were inhibited with specific sialyltransferase inhibitor or Tunicamycin which prevents N-glycosylation of fucosyltransferase. Tunicamycin has been shown to counteract GlcNAc from inducing the expression of E-cadherin and phosphorylation of β-catenin, it ultimately led to cell apoptosis. Therefore, the study emphasized the importance of *N*-glycosylation in cell survival [30]. Glycans have been recognized as important players in cell-cell interactions [31]. In cancer cells, malignant behaviors which depend on cell recognition- such as cell migration and survival- are mediated by distinct carbohydrate structures [1]. Since activated L1 can modulate sialylation and fucosylation, L1-induced specific glycosylation patterns may influence CHO cell survival and migration. This is in agreement with our previous studies in neuronal cells [3].

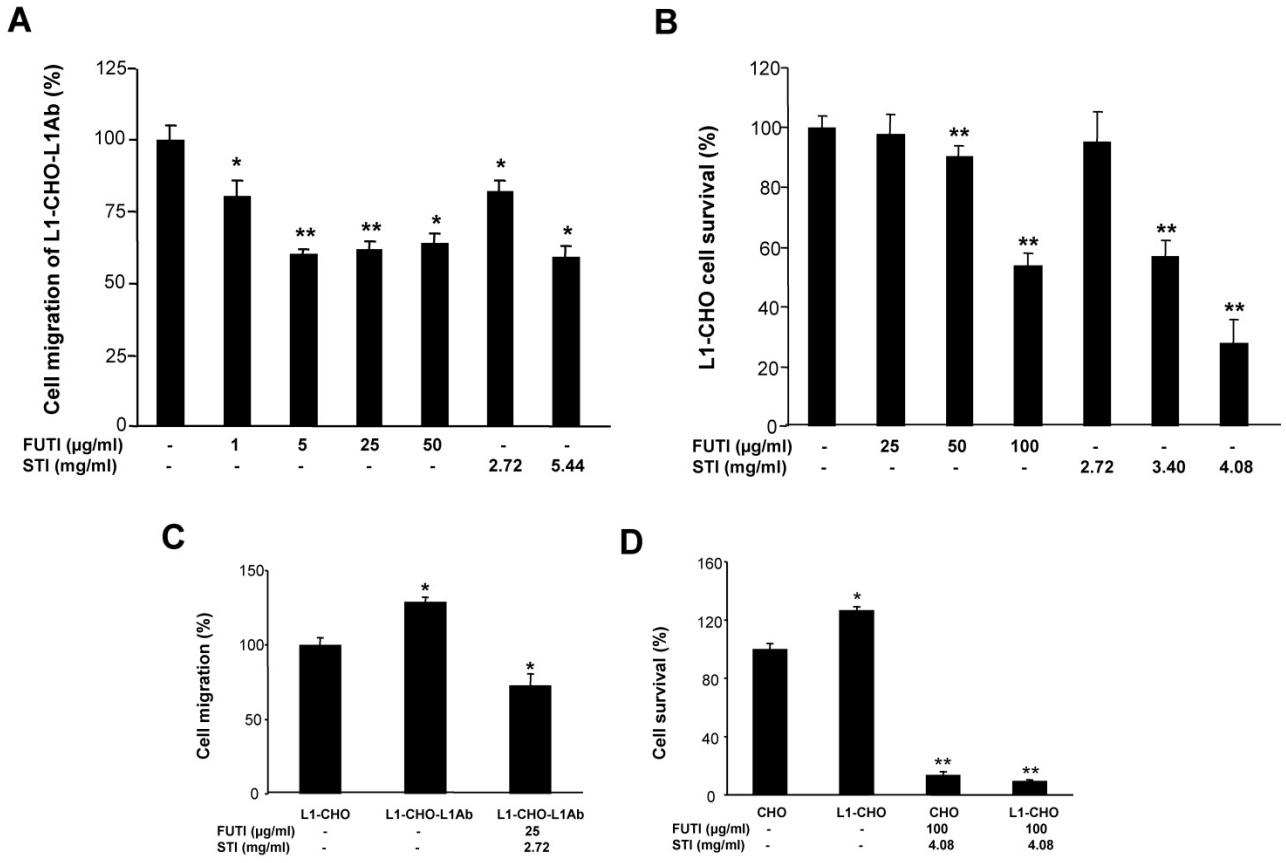

Figure 4. Inhibition of sialylation and fucosylation decreased cell migration and cell survival. A. Cell migration assay was performed in L1Ab-treated L1-CHO cells treated with Soyasaponin I (STI; 2.72mg/ml, 5.44mg/ml) or Tunicamycin (FUTI; 1μg/ml, 5μg/ml, 25μg/ml, 50μg/ml) versus L1-CHO. Cell migration was significantly inhibited after treatment with Soyasaponin I or Tunicamycin. **B.** MTT analysis of L1-CHO performed after treatment with Soyasaponin I (STI; 2.72mg/ml, 3.4mg/ml, 4.08mg/ml) or Tunicamycin (FUTI; 25μg/ml, 50μg/ml, 100μg/ml) showed a significant decrease of cell survival. **C.** Cell migration assay was performed in L1Ab-treated L1-CHO cells treated with Soyasaponin I (STI; 2.72mg/ml) and Tunicamycin (FUTI; 25μg/ml) together versus L1-CHO. Cell migration was significantly inhibited after treatment with Soyasaponin I and Tunicamycin together. **D.** MTT analysis of L1-CHO performed after treatment with Soyasaponin I (STI; 4.08mg/ml) and Tunicamycin (FUTI; 100μg/ml) together showed a significant decrease of cell survival. *: $p<0.05$; **: $p<0.01$, by Student's test.

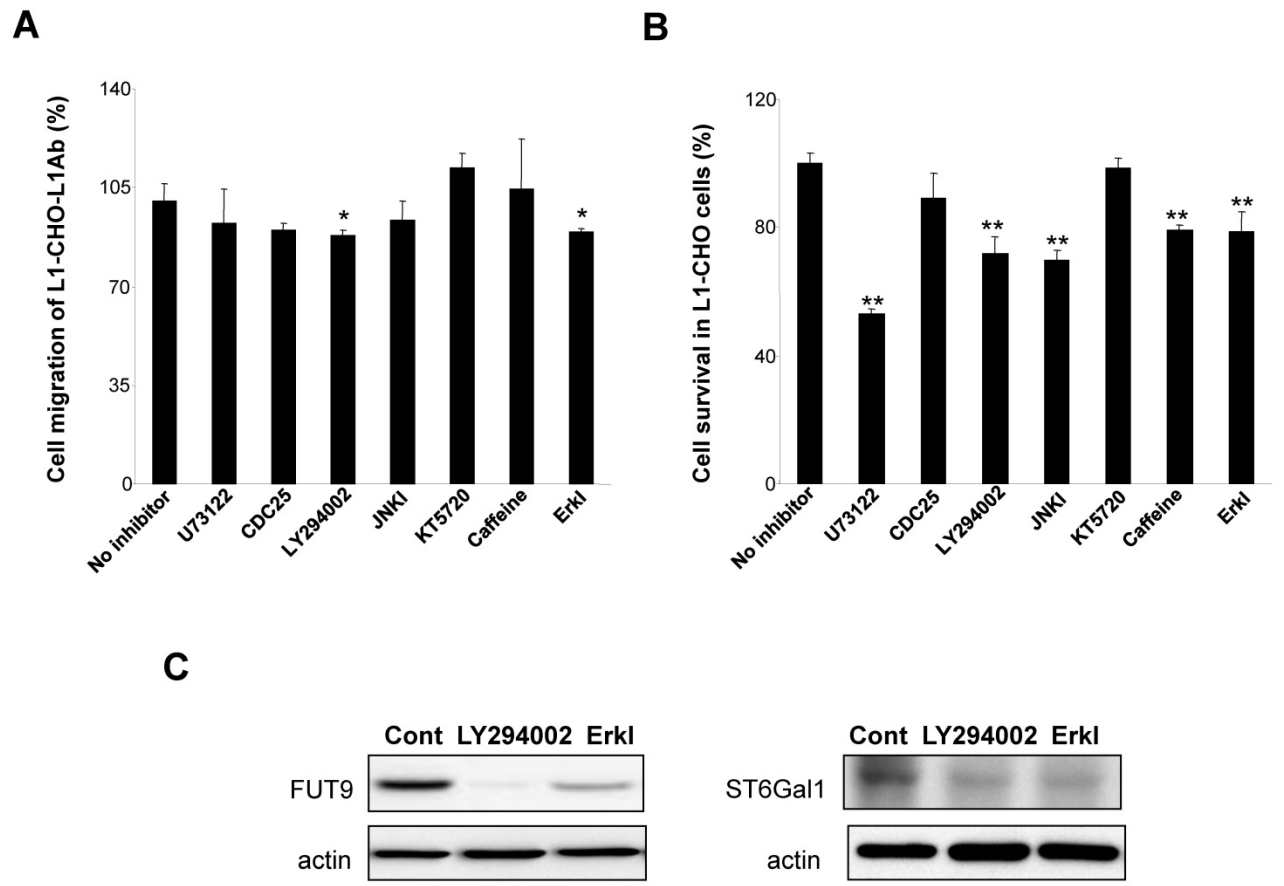

Figure 5. Effects of inhibitors of signal transduction pathways on cell migration, cell survival and the protein expressions of FUT9 and ST6Gal1. After incubation for 1 hour of L1-CHO cells treated with or without L1Ab, inhibitors of PLCγ (U73122, 10.5 μM), CDC25 phosphatase (CDC25 inhibitor II, 1.05 μM), PI3K (LY294002, 16.5 μM), Erk (Erk inhibitor, 50 μM), JNK (JNK inhibitor, 200nM), ATM/ATR (Caffeine, 1mM) or PKA inhibitor (KT5720, 280 nM) were added into the culture medium and the cells were incubated further for 24 hours. **A.** After treatment with inhibitors of the signal transduction pathways, cell migration assay was performed. Cell migration was significantly inhibited in L1Ab-treated L1-CHO cells by LY294002 and Erk inhibitor. **B.** After treatment with inhibitors of the signal transduction pathways, cell survival was quantified by MTT assay. U73122, LY294002, JNK inhibitor, Caffeine and Erk inhibitor significantly repressed cell survival in L1-CHO cells. **C.** Western blot was used to detect the expression of transferases. The protein expressions of ST6Gal1 and FUT9 were significantly downregulated in L1-CHO cells treated with LY294002 and Erk inhibitor. *: $p<0.05$; **: $p<0.01$, by Student's test comparison with the no inhibitor control.

L1 has been known to modulate the activation of AKT, MAPK, Erk, PI3K and FAK intracellular and extracellular signaling pathways [32-36]. To explore the mechanism by which L1 induces sialylation and fucosylation, we investigated the protein expressions of ST6Gal1 and FUT9 after cells were treated with inhibitors of signaling pathways known to be activated by L1. We show that L1 regulated sialylation and fucosylation to modulate CHO cell migration and survival via the PI3K-dependent and Erk-dependent pathways. The Erk pathway activated by L1 has been linked to a motile phenotype in carcinomas [37]. Additionally, Erk signaling has been reported to be involved in diverse cellular processes in cancer, including proliferation, survival, differentiation and migration [38, 39]. We propose that L1 may interact with transmembrane binding cell adhesion molecules, resulting in activation of Erk-dependent genes which induce cell surface sialylation and fucosylation and subsequently, promote cell migration and survival. PI3K/Akt signaling pathway has been reported to contribute significantly to cellular transformation and cancer development, and appears to have a serine/threonine kinase activity [40]. L1 has been reported to bind to integrin and PI3K is a classical component of the integrin signaling system. L1 acts as an integrin ligand, which activates the phosphorylation of downstream targets of the PI3K pathway. Therefore, the role of L1 in promoting cell migration and survival by regulating cell surface sialylation and fucosylation via the PI3K and Erk signaling pathways could be a promising therapeutic intervention in cancer.

Materials and Methods

Antibodies and inhibitors

Polyclonal goat anti-mouse, anti-rat and anti-human FUT9 antibodies and polyclonal goat anti-mouse, anti-rat and anti-human ST6Gal1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal

mouse anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA). R-Phycoerythin (R-PE)-conjugated mouse anti-rat monoclonal, PerCP-CY5.5-conjugated rat anti-mouse IgM monoclonal and R-PE-conjugated rat anti-mouse IgM monoclonal antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Biotinylated lectins and Texas Red avidin D were purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Soyasaponin I (Wako Pure Chem. Ind. Co. Ltd., Japan), Tunicamycin (Calbiochem, CA, USA), U73122 (Calbiochem, CA, USA), Cdc25 phosphatase inhibitor II (Calbiochem, CA, USA), LY294002 (Sigma, MO, USA), Erk inhibitor (3-(2-Aminoethyl)-5-((4-ethoxyphenyl) methylene) -2,4-thiazolidinedione, HCl; Calbiochem, CA, USA) and KT5720 (Sigma, MO, USA) are available commercially. L3, L4 and L5 antibodies were produced as previously described and presented by Melitta in Hamberg University (Kucherer *et al.*, 1987; Fahrig *et al.*, 1990; Streit *et al.*, 1990; Streit *et al.*, 1996) [42-45].

Cell culture

All cell culture reagents were purchased from Invitrogen Life Technologies (Merelbeke, Belgium) unless indicated otherwise. The CHO cells and CHO cells transfected with L1 were done as previously described [46]. The cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS, 0.5 U/mL penicillin and 0.5 U/mL streptomycin. Cells were passaged every 2 or 3 days using 0.05% trypsin/0.04% EDTA. The single cells obtained were used for flow cytometry assay.

Flow cytometry assay

Cell surface carbohydrate expression was assessed by indirect immunofluorescence detected by flow cytometry to provide a quantitative percentage binding and measure of the fluorescence intensity of carbohydrate antibodies and lectins. CHO cells were harvested into single cell suspensions by trypsinization as described above. Cells were placed in sterile conical tubes in aliquots of 500,000 cells each and stained with one of the 14 carbohydrate antibodies and lectins. Cells were washed 3 times with PBS and then stained with secondary antibody. Unstained cells and cells stained with secondary antibody alone were used as controls.

Migration assay

Costar transwell polycarbonate filters (5.0um pore size) were used for the migration assay [40]. The undersurfaces of the 6.5 mm transwell membranes were coated with anti-L1 antibody in PBS overnight at 4°C, then blocked with 2% BSA. Next 2.5x10E5 cells/mL were plated in culture medium into the upper chamber and allowed to migrate through the pores onto the coated undersurfaces at 37°C in a CO_2 incubator. After 24 hours, cells from the inner surface of the insert were gently wiped out with cotton-tipped swabs, and the inserts were fixed and stained. After a final wash with PBS, the cells were examined under a microscope to confirm proper morphology, and the dye was extracted. The absorbance was measured using a microplate reader. Dye levels are directly proportional to the numbers of cells. Data are presented as mean ± S.E.

MTT assay

Cells were cultured in 96 well plates for 24 hours. 10 μl 5mg/mL MTT solution was then added and cultured at 37°C for 4 hours. After washing with PBS, 20% SDS was added in to lyse the cells and dissolve the crystal. The OD (absorbance) was determined with aElx800 universal microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) and the percentage of cell survival was compared [47].

Western blot assay

Cells were harvested and lysed in RIPA buffer (1% Triton X-100, 150 mM NaCl, 25 mM Tris, pH 7.5, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM pyrophosphate, and 50 mM NaF). Samples containing equal amounts of protein were resolved using SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), followed by incubation with primary antibodies (anti-FUT9 antibody, anti-ST6Gal1 antibody, anti-PLCγ antibody) and secondary antibodies. Chemiluminescent detection was done using the ECL kit (Amersham Biosciences, Pittsburgh, PA, USA).

Data analysis

All data were expressed as mean ± S.E. Statistical evaluations were done by one-way analysis of variance and Student's t-test. Differences were considered to be significant when $p<0.05$.

Acknowledgements

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Competing Interests

The authors have declared that no competing interest exists.

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