

Lysophosphatidylserine Stimulates Chemotactic Migration of Colorectal Cancer Cells Through GPR34 and PI3K/Akt Pathway

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Abstract. *Background:* Lysophosphatidylserine (lysoPS) is a type of lysophospholipid mediator, which is involved in allergic conditions and tumor progression. We investigated the physiological function of lysoPS on colorectal cancer (CRC) cell lines, as well as the involved receptor and signaling pathways. *Materials and Methods:* Expression of lysoPS receptors on six cell lines was examined by reverse transcription-polymerase chain reaction (RT-PCR). The physiological functions of lysoPS were investigated, and experiments using small interfering RNA (siRNA) or inhibitors of the signaling pathways were conducted. *Results:* Among the three lysoPS receptors, GPR34 was highly expressed on all cell lines. LysoPS stimulated the chemotactic migratory ability. Wortmannin inhibited the migratory ability, as well as the GPR34 knock-down, strongly suggestive of the involvement of this receptor in the PI3K/Akt pathway. *Conclusion:* The involved receptor and pathways in the migratory ability in response to lysoPS was demonstrated, which opens premises for targeting as a new strategy for prevention and treatment of colorectal cancer.

Lysophosphatidylserine (lysoPS) is a type of lysophospholipid mediator, which is involved in allergic conditions and tumor progression (1). Other well-known lysophospholipids, such as lysophosphatidic acid (LPA) and sphingosine-1-phosphate (S1P), have lipid mediator-like actions (2-5) and are involved in blood vessel formation, lymphocyte egression from thymus, development of the brain and the cardiovascular system, and tumor growth or invasion

(4, 5). In contrast to S1P or LPA, which have been extensively investigated, the physiological functions of lysoPS have not been yet fully elucidated, especially in the field of oncogenesis and tumor metastasis.

LysoPS originates from phosphatidylserine (PS) by the hydrolysis of PS by the enzyme phosphatidylserine-specific phospholipase A1 (PS-PLA₁) (1). The highlighted function of lysoPS and PS-PLA₁ is the stimulation of histamine release from rat peritoneal mast cells, which indicates that lysoPS may be involved in allergic conditions (1). LysoPS and PS-PLA₁ are also involved in growth suppression of T-cells (6) and neural cell differentiation (7). As for oncogenesis or tumor metastasis, lysoPS stimulates chemotactic migration of glioma or fibroblast cell lines (8, 9). Among the three human lysoPS receptors, namely GPR34, P2Y10 and GPR174, the former has been shown to play an important role in the tumor progression of gastric cancer (10). Also, expression of PS-PLA₁ has been shown to correlate with metastasis of human melanoma cell lines (11). Recently, we found that expression of PS-PLA₁ on human colorectal cancer (CRC) tissue also correlates with tumor invasion and metastasis (article submitted for publication).

These studies suggest that lysoPS and its producing enzyme, PS-PLA₁, play an important role in tumor cell migration and metastasis. In the present study, we investigated the physiological function of lysoPS on colorectal cancer cell lines and the signaling pathways involved.

Materials and Methods

Materials. L- α -lysophosphatidylserine was purchased from Avanti Polar Lipid (Alabaster, AL, USA). Pertussis toxin (PTX) and genistein were from Sigma (St Louis, MO, USA). RPMI-1640 cell culture medium, bovine serum albumin (BSA), fatty acid free bovine serum albumin (FA(-)BSA), antibiotics/antimycotics and wortmannin were from Sigma-Aldrich (St. Louis, MO, USA). Fetal calf serum (FCS) was from Gibco BRL (Grand Island, NY, USA). Calcium and magnesium-free phosphate-buffered saline (PBS(-)) was from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

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Cell culture. The human colorectal cancer cell lines, SW480, LoVo, HT29, WiDR, DLD1 and Caco2, obtained from the Japanese Cancer Research Resource Bank, were used. Cells were cultured in RPMI-1640 medium supplemented with 5% fetal calf serum and 1% antibiotics/antimycotic and incubated in a 5% CO₂ incubator at 37°C.

RT-PCR analysis. Cells cultured on 6-well dishes were used for reverse transcription polymerase chain reaction (RT-PCR) analysis. mRNA of colorectal cancer cell lines were isolated by using RNeasy Mini Kit (Qiagen, Austin, TX, USA). cDNA were obtained from mRNA by using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster, CA, USA). The sequences of the primers used were as follows: GPR34: forward, 5'-GGG ACT GGT TGG GAA CAT AA-3'; reverse, 5'-GAA AGG GAG GCA GAA GAT GA-3'. P2Y10: forward, 5'-GCA GGA TTT GTG ATC CCA GT-3'; reverse, 5'-CCT CTC ACT GAT CCC TTG GA-3'. GPR174: forward, 5'-TTC CTT GCC ACT GAG GAT CT-3'; reverse, 5'-AAA ATC GTC GCA CAC TGA TG-3'. Primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were purchased from Operon. Amplification was performed over 40 cycles (95°C/1 min for denaturation, 55°C/1min for annealing and 72°C/1 min for extension) by using GoTaq Green Master Mix (Promega, Fitchburg, WI, USA). PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

Western blot analysis of p-Akt and GPR34. Cells cultured on 6-well dishes were used for western blot analysis. After washing three times with PBS(-), the cells were lysed with 0.3 ml of Tris-saline [50 mM Tris-HCl (pH7.6), 150 mM sodium chloride] containing various protease inhibitors (1 mM ethylene glycol tetraacetic acid (EGTA), 0.1 mM diisopropyl fluorophosphates, 0.5 mM phenylmethylsulphonyl fluoride, 1 mg/ml Na-r-tosyl-L-lysine chloromethyl ketone, 1mg/ml antipain, 0.1 mg/ml pepstatin, 1mg/ml leupeptin) and 1% Triton-X for 1h in a cold room and subsequently cells were harvested by scraping the culture dishes with a cell scraper. After centrifuging at 15,000 rpm for 5 min, the clear supernatant was collected and used for the cell protein extract. Protein concentration was determined using the BCA Protein Assay Kit (Pierce Biomedical Co, Rockford, IL, USA). Cell lysates were electrophoresed on any kD Mini-PROTEAN TGX GEL (Bio-Rad, Hercules, CA, USA) and were transferred onto Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% skim milk for 30 min and then incubated with each primary antibody (anti-β-actin antibody (Santa-Cruz Biotechnology, Dallas, Texas, USA), anti-GPR34 antibody (R&D Systems, Minneapolis, MN, USA) or anti-phospho-Akt antibody (Cell Signaling Technology, Danvers, MA, USA)) overnight at 4°C. The membrane was washed and incubated with 1:10000 dilution of biotinylated anti-mouse IgG or anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA, USA) for 60 min at room temperature. The immunoreactive bands were visualized by chemiluminescence using the ECL detection system (Amersham Pharmacia Biotech).

Migration assay. Chemotactic or chemokinetic migration of cells in response to a gradient concentration of lysoPS was measured in a modified Boyden Chamber assay. In brief, a polycarbonate filter with 8-μm pores (Neuro Probe, Gaithersburg, MD, USA), which was coated with collagen type I, was placed on a 96-well chamber (Neuro Probe) containing 0.1 to 50 μM lysoPS on the lower chamber or on both

chambers. SW480 or LoVo cells (5×10⁵ cells in 200 μl/well) were loaded into the upper chamber. Solutions with lysoPS and the cell suspension were prepared in RPMI1640 containing 0.1% FA(-)BSA. Some cells were pretreated with 100 ng/ml PTX for 4 h, 400 nM wortmannin for 1h or 200 μM genistein for 1h. After incubation at 37°C for 6 h, the filter was disassembled and the cells on the filter were fixed with 99% methanol and stained with the Diff-Quick staining kit (Sysmex Co., Kobe, Japan). The upper side of the filter was scraped to eliminate the non-migrated cells. The number of the cells that migrated to the lower side of the filter was measured by reading the staining intensity using a 96-well microplate reader at 595nm.

Proliferation assay. The Cell Titer 96® aqueous nonradioactive cell proliferation assay (Promega, Madison, WI, USA) was used for the assessment of cell proliferation. SW480 cells or LoVo cells were plated in 96-well plates (5000 cells/well). After a 24 h-culture at 37°C to allow cells to adhere and stabilize, lysoPS (0.01 to 20 μM) was added and cells were cultured for another 72h. Subsequently, 20 μl of proliferation assay solution was added to each well and, after incubated at 37°C for 3 h, the absorbance was measured at 490 nm in a 96-well plate using a microtiter plate reader (Beckton Dickinson). The proliferation of the cells was calculated as the ratio of each experimental well to the control.

Flow cytometric analysis of integrin-β1 expression. SW480 or LoVo cells, treated with lysoPS (1 to 10 μM), were incubated with mouse antibody against β1 integrin or mouse IgG (5 μg/ml; BD Pharmingen, San Diego, USA) for 30 min at 4°C. Then the cells were incubated with FITC-labeled secondary antibodies (10μg/ml; BD Pharmingen) for 30 min at 4°C and analyzed in a flow cytometer (Becton-Dickinson).

Adhesion assay. The wells of 96-well plates were pre-coated with collagen type I (30 μg/ml; Nitta Gelatin, Osaka, Japan). After blocking of nonspecific binding with RPMI-1640 containing 0.1% FA(-)BSA, SW480 or LoVo cells, previously stained with Calcein (Dojindo, Kumamoto, Japan) and then stimulated with 1 to 25 μM lysoPS for 1 h, were seeded (1×10⁵ cells/well) and incubated at 37°C for 15 min. After washing with PBS to remove non-adherent cells, the fluorescence of the adhered cells was measured in a fluorometer (TERASCAN VP; Minerva Tech, Tokyo, Japan). The adhesion rate was calculated as the ratio between experimental wells and positive wells.

GPR34 knock-down using siRNA. Small interfering RNA (siRNA) that specifically target the GPR34 transcript was purchased from Santa Cruz Biotechnology. siRNA was transfected into SW480 cells according to the manufacturer's instructions. Cells were used for RT-PCR, Western blot analysis or migration assay 72 h after the transfection of siRNA.

Statistical analysis. All experiments were repeated at least three times. Statistical significance of the difference was evaluated by the non-paired, two sided Student's *t*-test. Two sided *p*-value less than 0.05 were regarded as statistically significant.

Results

Expression of lysoPS receptors on colorectal cancer cell lines. The expression of lysoPS receptor mRNA on colorectal cancer cell lines was examined by RT-PCR (Figure

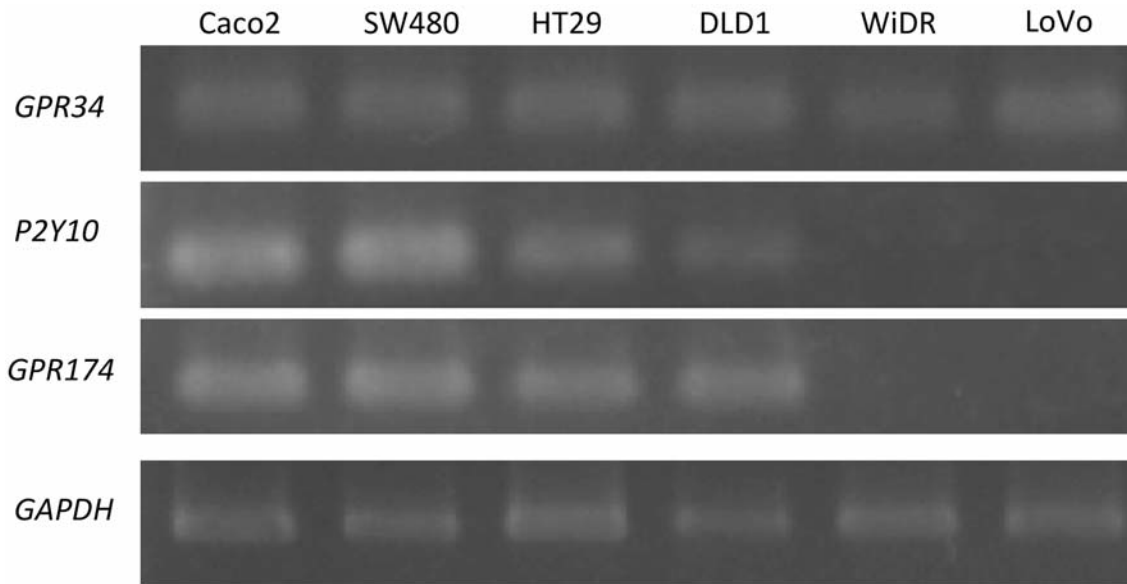


Figure 1. RT-PCR analysis of lysoPS receptor expression on colorectal cancer cell lines. RNA extracted from the colorectal cancer cells were analyzed by RT-PCR. Expression of GAPDH was used as the internal control.

1). Among the three lysoPS receptors known to be expressed on human tissues, namely GPR34, P2Y10 and GPR174 (12), the former was highly expressed on all colorectal cancer cell lines, whereas in two of them (WiDR and LoVo) the P2Y10 and GPR174 expression was not detected. SW480 expressed all of them, while LoVo expressed only GRP34. Since a different receptor expression was observed between SW480 and LoVo, these cells were selected for the functional experiments, in which we aimed to determine the receptors and the signaling pathways involved in response to lysoPS stimulation.

LysoPS stimulates chemotactic and chemokinetic migration of SW480 and LoVo. As shown in Figure 2A, 2B, lysoPS induced both chemotactic and chemokinetic migration of SW480 cells with maximum stimulation at 5-10 μ M (2.5 ± 0.1 -fold increase at 10 μ M compared to control). The migratory ability decreased with further increase of the lysoPS concentration, giving a typical bell-shaped curve for both chemotactic and chemokinetic migration. A similar effect of lysoPS was observed with LoVo (Figure 2C, 2D). LysoPS stimulated the chemotactic and chemokinetic migration of both SW480 and LoVo, indicative that lysoPS exerts its function through GPR34, which is expressed on both cell lines.

LysoPS does not promote the proliferative ability nor affects the ability of colorectal cancer cells to adhere to extracellular matrix proteins. As shown in Figure 3, the

proliferative ability of SW480 and LoVo was almost not affected by lysoPS at 0.01 to 1 μ M, but exposure to higher doses resulted in suppression of the proliferative activity of both cell lines. It indicates that the proliferative ability of SW480 and LoVo are not promoted by lysoPS.

Next, we investigated the expression level of β 1-integrin, which exists in dimer complexes with various α -chains and is an important receptor for the adhesion of cells to the extracellular matrix proteins, namely collagens, laminin and fibronectin. We observed that the β 1-integrin expression was not affected by the lysoPS treatment on either SW480 or LoVo cells (data not shown). This finding was also confirmed by the adhesion assay, in which the ability of both cell lines to adhere to collagen type I was not affected by lysoPS (data not shown).

LysoPS stimulates cell migration through the PI3K/Akt pathway. To understand the downstream signaling pathways of lysoPS leading to chemotactic migration of SW480, we tested several agents that disturb signal transduction of G protein (Gi), PI3K/Akt, and p38 MAPK pathways. For this purpose, cells were pre-treated with either PTX (100 ng/ml) for 4 h, wortmannin (400 nM) for 1 h or genistein (200 μ M) for 1 h, respectively, then stimulated with lysoPS and the migratory activity was analyzed. As shown in Figure 4A, PTX, which disturbs the signal transduction of G protein (Gi) and wortmannin, a PI3K inhibitor, significantly suppressed the chemotactic migration induced by lysoPS. On the other hand, genistein, a tyrosine kinase inhibitor known to inhibit the

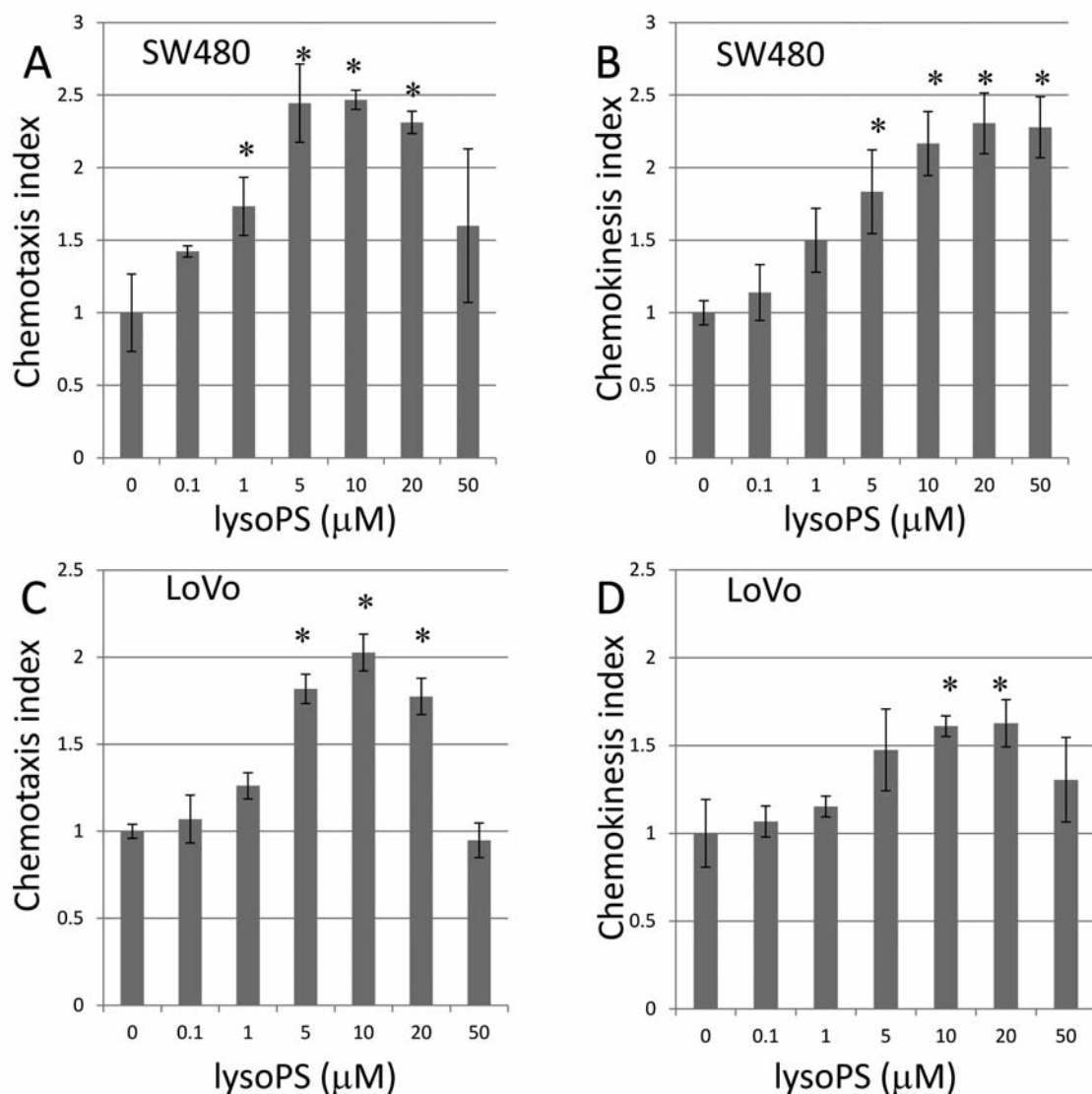


Figure 2. A migration assay showing the effect of lysoPS on SW480 or LoVo. Cancer cells (5×10^5 cells in 200 μ l) were added to the upper well of a 96-well chamber and assessed for migration across a polycarbonate membrane with 8- μ m pore in the presence of various concentrations of lysoPS for 6 h at 37°C. Chemotaxis (2A; SW480, 2C; LoVo) and Chemokinesis (2B; SW480, 2D; LoVo) in response to lysoPS were analyzed. Columns indicate the mean of three studies performed in triplicate and bars indicate S.D. *indicates significant difference from the control ($p < 0.05$).

function of MAPK, had almost no effect on the chemotactic migration induced by lysoPS. These findings indicate that the PI3K/Akt pathway signaling is the major pathway involved in the chemotactic migration of SW480 induced by lysoPS. To confirm this, we next analyzed the phosphorylation of Akt by western blot and found a transient increase of Akt phosphorylation by the treatment of SW480 cells with lysoPS (10 μ M), with the maximal effect being observed 5-10 min after the stimulation (Figure 4B). Thus, we confirmed the PI3K/Akt pathway as the main mechanism involved in the chemotactic migration of SW480 induced by lysoPS.

GPR34 knock-down inhibits the migration of SW480 induced by lysoPS. To confirm the involvement of *GPR34* in the chemotactic migration of SW480 induced by lysoPS, experiments using SW480 cells with *GPR34* knock-down, by means of the si-RNA, were conducted. Figure 5A shows the successful suppression of the *GPR34* mRNA (58% decrease) and *GPR34* protein (48% decrease) on knock-down cells. These cells were tested in the migration assay, as above described. In mock-transfected cells, lysoPS stimulated the chemotactic migration, but it was almost completely abolished in *GPR34* knock-down cells (Figure 5B). Thus, we

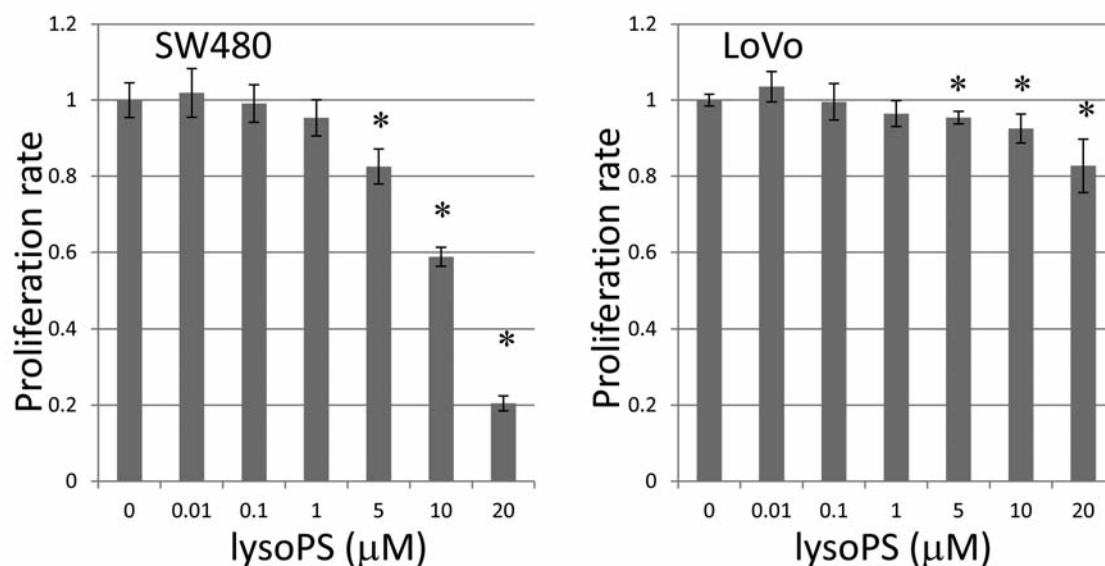


Figure 3. A proliferation assay showing no stimulatory effect of lysoPS on SW480 or LoVo. Cancer cells were treated with various concentrations of lysoPS for 72 h and the proliferation rate was analyzed. Columns indicate the mean of three studies performed in triplicate and bars indicate S.D. *indicates significant difference from the control ($p < 0.05$).

confirmed the involvement of GPR34 receptor in the chemotactic migration induced by lysoPS.

Discussion

Presently, only few reports exist related to the physiological function of lysoPS on tumor cells. LysoPS has been shown to stimulate the chemotactic migration of glioma and fibroblast cell lines (8, 9) and its receptor, GPR34, to play an important role in tumor progression of gastric cancer (10). As for the lysoPS-producing enzyme, PS-PLA₁, although an inverse correlation was reported between its expression and the metastatic potential in melanoma cells (11), we found a positive correlation with tumor invasion and metastasis in colorectal cancer by immunohistochemistry (article submitted for publication). From these facts, we speculated that lysoPS and PS-PLA₁ might play an essential role in colorectal cancer cell migration and metastasis.

To confirm this hypothesis, we evaluated the effect of lysoPS on the migratory activity of colorectal cancer cells, namely SW480 and LoVo, with distinct expression patterns of lysoPS receptors and observed that both lines exhibited increased chemotactic and chemokinetic migratory activities when stimulated with lysoPS, without promoting the proliferative ability nor affecting the ability to adhere to collagen. The results of the migration assay are compatible with previous reports showing the increased migratory activity of glioma or fibroblast cells treated with lysoPS (8, 9). Since a similar increase of the migratory activity by

lysoPS was observed in both SW480 and LoVo cells, and the common lysoPS receptor expressed on both cells was the GPR34, we speculated that this is the main receptor involved in colorectal cancer cell migration induced by lysoPS. Using inhibitors of various intracellular signaling pathways, we found that the stimulation of the chemotactic migration of SW480 induced by lysoPS was mainly dependent on the PTX-sensitive G protein (Gi), which is a component of GPR34 and the PI3K/Akt, but not on the MAPK pathway. In glioma cells, however, the chemotactic migration induced by lysoPS was shown to be dependent on both the PI3K/Akt and the MAPK pathways (8), a disparity probably dependent on the difference of lysoPS receptor expressions. Also, we confirmed the increased phosphorylation of Akt by the treatment of cells with lysoPS, corroborating the above findings.

Next, to confirm the involvement of GPR34 in the lysoPS-induced chemotactic migration of SW480, knock-down experiments, by means of si-RNA, were conducted. We clearly demonstrated that the migratory activity of colorectal cancer cells induced by lysoPS was significantly inhibited in GPR34 knock-down cells compared to mock-transfected ones. Together with the report on the association of GPR34 with tumor progression in gastric cancer (10), our present results indicate the importance of GPR34 in the progression, not only of gastric, but also colorectal cancers. To the best of our knowledge, this is the first report to show the involvement of GPR34 in lysoPS-induced chemotactic migration of cancer cells.

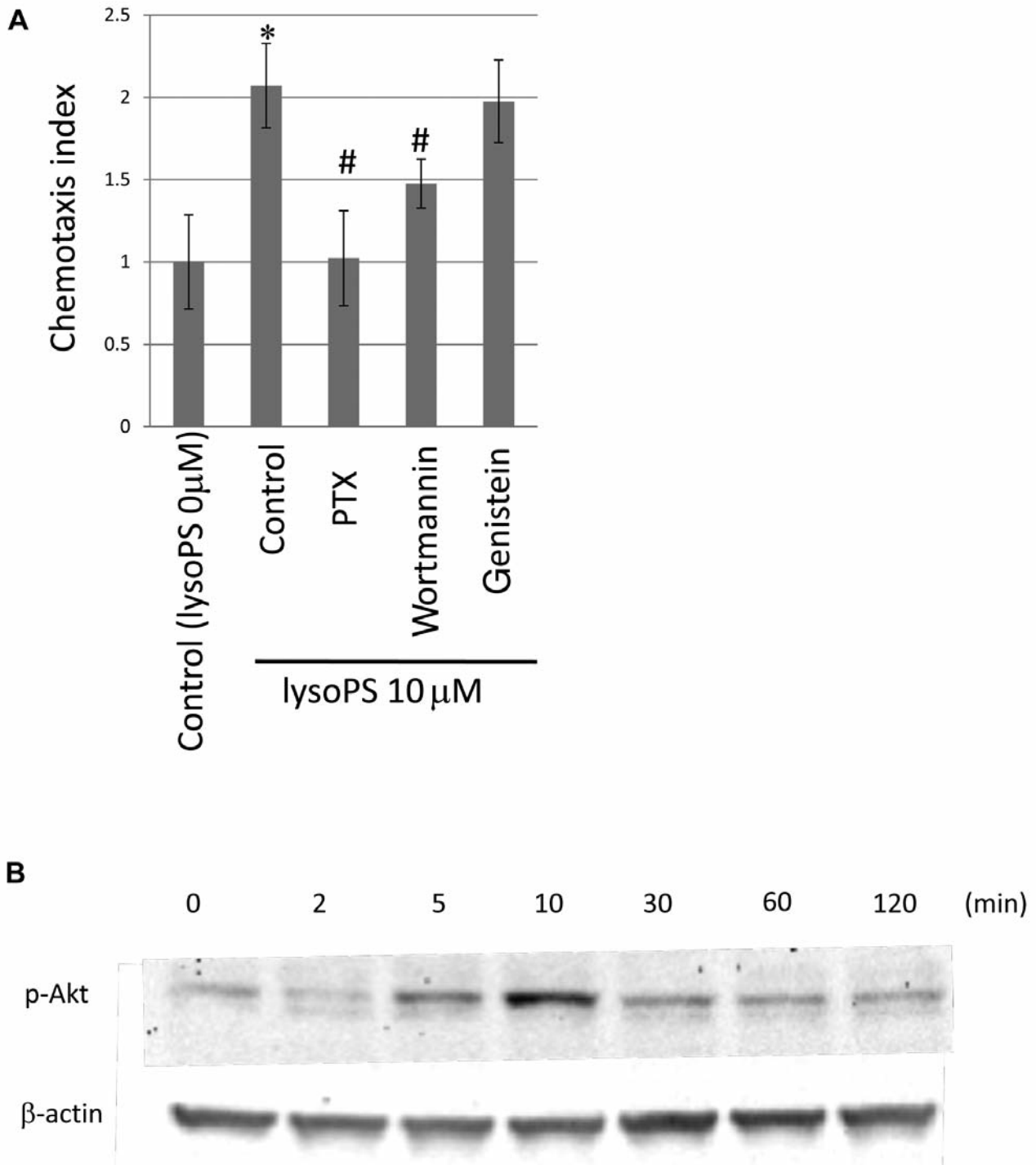


Figure 4. A. A chemotactic migration assay showing the effect of several inhibitors of signal transductions on lysoPS-induced chemotactic migration of SW480. Cell migration induced by lysoPS (10 μM) was assayed in SW480 cells pre-treated with or without PTX (100 ng/ml, 4h), which inactivates Gi protein, wortmannin (400 nM, 1h), which inactivates PI3K/Akt pathway, or genistein (200 μM, 1h), a MAPK inhibitor. Columns indicate the mean of three studies performed in triplicate and bars indicate S.D. *indicates significant difference from the control (lysoPS 0 μM) (p<0.05). #indicates significant difference from the control (lysoPS 10 μM, without inhibitors). B. Western blot analysis showing the expression of pAkt (phospho-Akt) stimulated by lysoPS. SW480 cells were incubated with 10 μM of lysoPS for the times indicated. Extracts from the cells (7.5 μl/lane) were prepared and analyzed for expression of pAkt levels, which were determined using anti-phospho-Akt antibody. The most prominent increase of pAkt expression was observed after 5-10 min treatment with lysoPS.

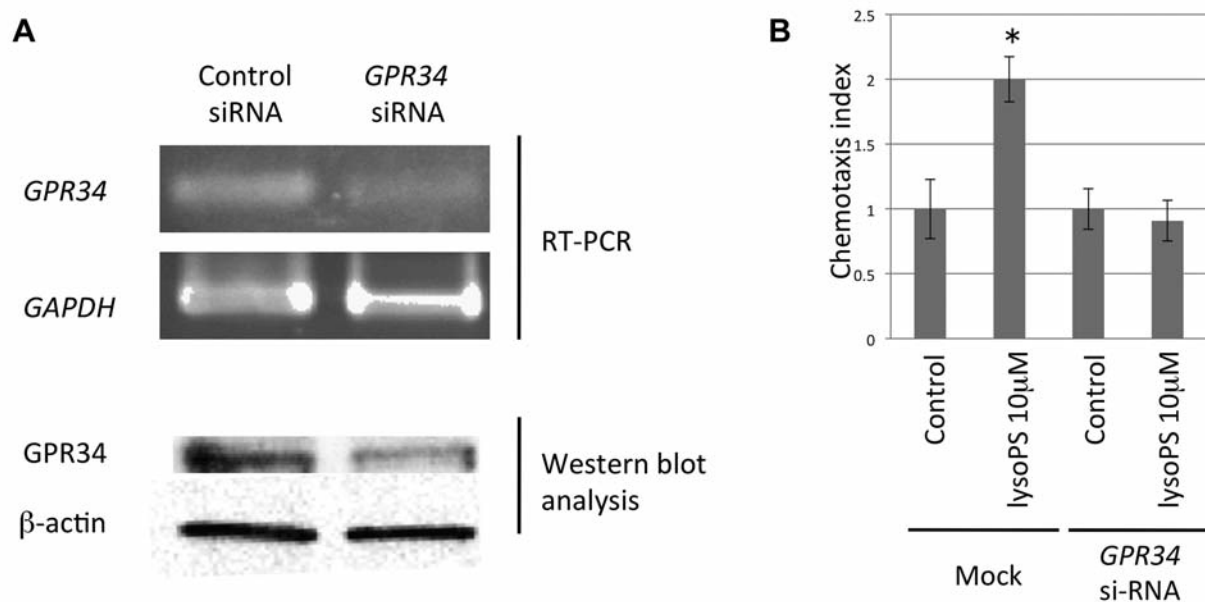


Figure 5. A. RT-PCR and Western blot analysis showing down-regulation of GPR34 by siRNA. SW480 cells were incubated with either control si-RNA or GPR34 si-RNA, and extracts from the cells were analyzed by RT-PCR and western blot analysis. The important inhibition of GPR34 on the GPR34 knock-down SW480 cells was confirmed by both methods. B. A chemotactic migration assay showing the effect of lysoPS on GPR34 knock-down SW480 cells. Both GPR34 knock-down (GPR34 si-RNA) and mock-transfectant (Control si-RNA) SW480 cells were induced to migrate in the presence of lysoPS (10 μM). Compared to mock-transfectants, the migratory ability of GPR34 knock-down cells was significantly inhibited. Columns indicate the mean of three studies performed in triplicate and bars indicate S.D. *indicates significant difference from the control ($p < 0.05$).

LysoPS originates from PS (phosphatidylserine) by the hydrolysis of PS by the enzyme PS-PLA₁ (1). PS is normally restricted to the inner surface of the cellular membrane and translocates to the cellular surface in case of apoptosis or cell activation (13). PS exposure is enhanced in response to chemotherapy or radiotherapy (14, 15) and abundant lysoPS may be produced by PS-PLA₁ in such conditions. Since chemotherapy and chemoradiotherapy are becoming standard modalities in the management of CRC, especially those far advanced with distant metastasis or locally or distally recurred after the curative treatment (16), the cascade of PS - PS-PLA₁ - lysoPS would be an important target for the development of new therapeutic strategies.

In conclusion, the stimulatory effect of lysoPS on colorectal cell migration and the involvement of GPR34, PI3K/Akt pathway in this process were demonstrated. Further studies are necessary for the development of strategies to target this cascade for the improvement of the therapeutic strategies for colorectal cancer, as well as other tumor types.

Conflicts of Interest

All Authors have no conflict of interest to declare.

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