

Piperlongumine inhibits gastric cancer cells via suppression of the JAK1,2/STAT3 signaling pathway

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Abstract. Piperlongumine (PL), a major active component of long peppers, has been reported to possess anti-cancer properties; however, its effect on gastric cancer (GC) has remained to be demonstrated. The present study assessed the effects of PL on the MKN45 and AGS GC cell lines and explored the underlying mechanisms. An MTT assay revealed that PL suppressed the proliferation of GC cells, while flow cytometric analysis showed that PL inhibited cell cycle progression. Furthermore, Transwell assays revealed the inhibitory effects of PL on the invasion and migration of GC cells. In addition, PL reduced the phosphorylation of Janus kinase (JAK)1, JAK2 and signal transducer and activator of transcription (STAT)3 in a concentration-dependent manner, as indicated by western blot analysis, and decreased the expression of STAT3-dependent tumor-associated genes in GC cells, as revealed by PCR analysis. In conclusion, the present study was the first, to the best of our knowledge, to reveal the efficacy of PL against GC. The consumption of long peppers is therefore recommended for the prevention and treatment of GC, and PL may be a promising candidate drug for treating GC.

Introduction

Gastric cancer (GC), one of the most common gastrointestinal malignancies in the world, accounts for ~8% of all cancers (1) and is the second leading cause of cancer-associated mortality (2). Highest incidence rates are in Eastern Asia, Eastern Europe and South America, and lowest incidence rates are in North America and the majority of Africa (1). The

genesis and progression of GC are multi-step and multi-stage processes. In spite of the treatments available for GC, including surgery, radiotherapy, chemotherapy and gene therapy (3-8), the outcome is dismal due to relapses originating from the residual nidus being common. To improve the outcome for patients with GC and enhance the efficacy of GC treatments, novel drugs are urgently required.

Piperlongumine (PL), a primary constituent of long peppers, is a natural alkaloid found in the fruit as well as the roots of the plant (9). PL has broad biological activities, including bactericidal and insecticidal capabilities (10). Furthermore, it exhibits anti-atherosclerotic, anti-inflammatory, anti-platelet, cardioprotective, anti-depressant and analgesic effects (11-17). Of note, accumulating evidence has shown that PL has anti-cancer properties. For example, Randhawa *et al* (18) reported that PL could suppress the proliferation of colon cancer cells in a concentration- and time-dependent manner through the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway. Furthermore, Dhillon *et al* (19) demonstrated that PL inhibited the proliferation of pancreatic cancer cells by upregulating the levels of reactive oxygen species, which caused DNA damage. In addition, PL was reported to have anti-proliferative and apoptosis-inducing effects on human ovarian cancer cells (20).

However, to the best of our knowledge, the effects of PL on GC cells have not been demonstrated to date. Therefore, the present study evaluated the effects of PL on the MKN45 and AGS GC cell lines and explored the underlying mechanisms. The results demonstrated that PL inhibited the proliferation, cell cycle progression as well as cell invasion and migration of GC cells through suppression of the Janus kinase (JAK)1,2/signal transducer and activator of transcription (STAT)3 signaling pathway.

Materials and methods

Cell lines and reagents. The MKN45 and AGS human GC cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaille, France) in a humidified atmosphere containing 5% CO₂ at 37°C. PL was purchased from Sigma-Aldrich (St. Louis, MO, USA).

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Cell proliferation assay. Cell proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, MKN45 and AGS cells were seeded into 96-well plates at a density of 1×10^4 cells/well and cultured for 24 h. The cells were then treated with various concentrations of PL (0, 10, 20 or 40 μM) for 24, 48, 72 or 96 h. Subsequently, 20 μl MTT solution (Sigma-Aldrich) was added to each well, followed by incubation at 37°C for 4 h. The medium was carefully removed and 100 μl dimethyl sulfoxide (Sigma-Aldrich) was added into each well. The absorbance at 490 nm was determined using a spectrophotometer (GENESYS™ 20; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Each experiment was performed at least three times.

Cell cycle assay. After treatment for 24 h with PL (0, 10, 20 or 40 μM), the cells were trypsinized and fixed overnight in 70% ethanol (Sigma-Aldrich) at -20°C. Next, the cells were collected and re-suspended in staining solution containing 50 mg/l propidium iodide (Sigma-Aldrich) and 100 mg/l RNase A (Sigma-Aldrich), followed by incubation in the dark for 30 min at room temperature. The cell cycle distribution was then analyzed with a flow cytometer (BD FACSAria II; BD Biosciences, Franklin Lakes, NJ, USA).

Cell invasion and migration assays. The invasive and migratory capacity of GC cells was detected using 24-well Transwell chambers (containing filters with 8- μm pore size; Corning, Inc., Corning, NY, USA) according to the manufacturer's instructions. For the invasion assay, the insert membrane was coated with Matrigel (Invitrogen; Thermo Fisher Scientific, Inc.), while it was kept in its original condition for the migration assay. MKN45 or AGS cells (1×10^5) were seeded in the upper chambers and cultured in serum-free medium containing PL (0, 10, 20 or 40 μM) for 24 h. Culture medium containing 10% FBS was added to the lower chamber. After 24 h of incubation at 37°C, the cells on the upper surface of the membrane were scraped off with cotton swabs, while the cells on the lower surface of the membrane were fixed with methanol (Sigma-Aldrich) and stained with 0.1% crystal violet (Sigma-Aldrich), followed by counting under a microscope (CX22; Olympus Corporation, Tokyo, Japan). At least three independent experiments were conducted.

Western blot analysis. MKN45 cells were treated with 0, 10, 20 and 40 μM PL for 24 h and then lysed at 4°C for 20 min in radioimmunoprecipitation assay buffer (Sigma-Aldrich) containing 1% Nonidet P40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1 μM sodium orthovanadate, 0.03% aprotinin and 10 ng/ml phenylmethylsulfonyl fluoride. The total protein was separated by 10% SDS-polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA) and then transferred onto a polyvinylidene difluoride membrane (Pierce Biotechnology, Inc., Rockford, IL, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing Tween 20 (TBST; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 1 h prior to incubation with the primary antibodies as follows: Rabbit polyclonal anti-JAK1 (1:1,500; Cell Signaling Technology, Inc., Danvers, MA, USA; cat.

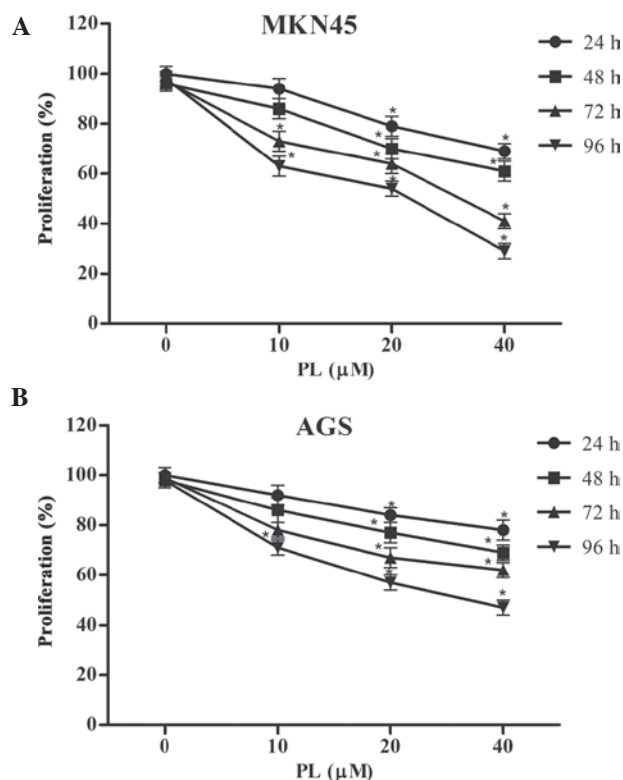


Figure 1. PL suppresses the proliferation of gastric cancer cells. (A) MKN45 and (B) AGS cells were treated with 10, 20 or 40 μM of PL for 24, 48, 72 or 96 h and cell proliferation was quantified using the MTT assay. Values are expressed as the mean \pm standard deviation of the percentage of the control group from three individual experiments. *P<0.05 compared with the control group. PL, piperlongumine.

no. 3332); rabbit polyclonal anti-phosphorylated (p)-JAK1 (1:1,500; Cell Signaling Technology, Inc.; cat. no. 3331); rabbit polyclonal anti-JAK2 (1:1,500; Cell Signaling Technology, Inc.; cat. no. 3773); rabbit polyclonal anti-p-JAK2 (1:1,500; Cell Signaling Technology, Inc.; cat. no. 3774); mouse monoclonal anti-STAT3 (1:1,500; Cell Signaling Technology, Inc.; cat. no. 9139), mouse monoclonal anti-p-STAT3 (1:1,500; Cell Signaling Technology, Inc.; cat. no. 9138); and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight. Following washing with TBST for 10 min, the membranes were incubated for 1 h at room temperature in goat anti-mouse horseradish peroxidase-conjugated (1:3,000; Santa Cruz Biotechnology, Inc.; cat. no. sc-2302) and rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody (1:2,000; Santa Cruz Biotechnology, Inc.; cat. no. sc-358920) and then washed with TBST three times. The protein bands were visualized using a Pierce ECL Western Blotting kit (Thermo Fisher Scientific, Inc.). The absorbance values of target proteins were analyzed with Gel-Pro Analyzer software (version 4.0; Media Cybernetics, Inc., Rockville, MD, USA).

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis. The total RNA was isolated from the PL-treated MKN45 cells with TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions and then transcribed into complementary DNA using a PrimeScript RT reagent kit (Takara Bio Inc., Otsu, Japan).

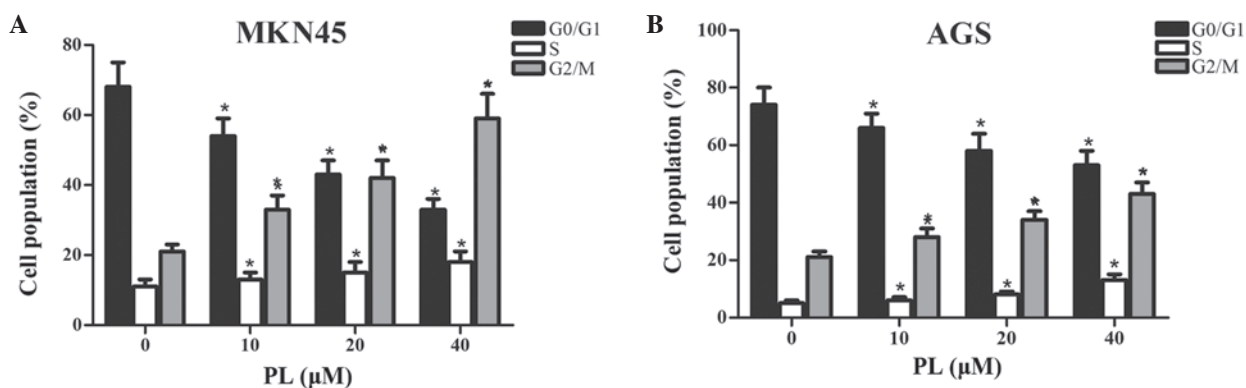


Figure 2. PL induces cell cycle arrest in gastric cancer cells. Flow cytometry was applied to analyze the cell cycle distribution of (A) MKN45 and (B) AGS cells treated with different concentrations of PL for 24 h. Values are expressed as the mean \pm standard deviation of the percentage from three individual experiments. *P<0.05 compared with the control group. PL, piperlongumine.

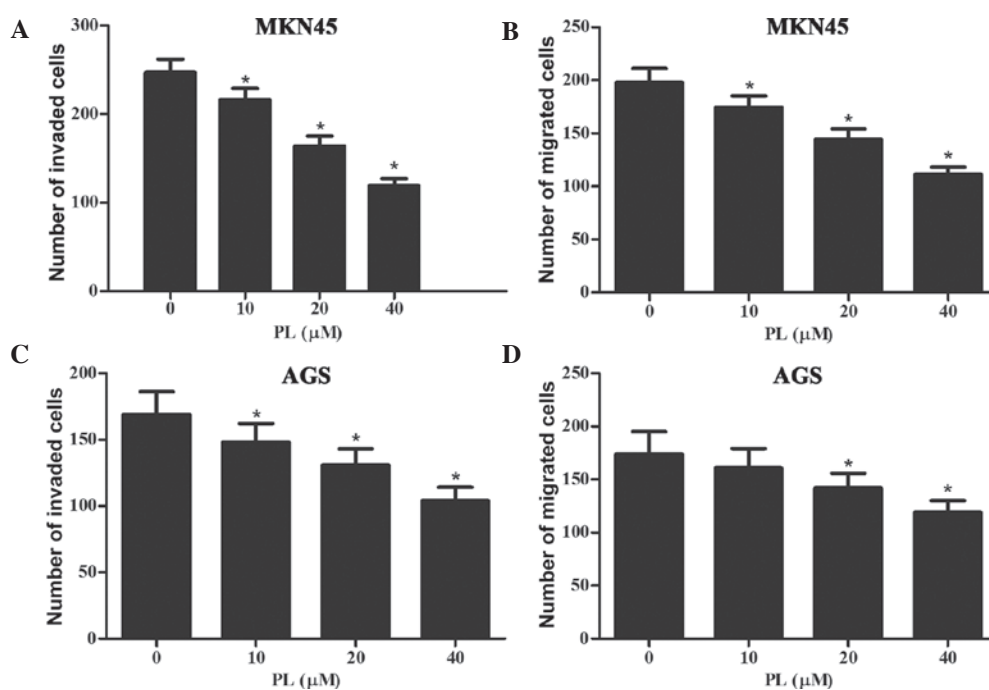


Figure 3. PL suppresses the invasion and migration of gastric cancer cells. Cells were treated with 0, 10, 20 or 40 μ M PL for 24 h, followed by assessment of invasion and migration using Transwell chambers. In MKN45 cells treated with PL, the amount of cells (A) invading and (B) migrating through the membrane was decreased. In AGS cells treated with PL, the amount of cells (C) invading and (D) migrating through the membrane was also decreased. Values are expressed as the mean \pm standard deviation of number of cells per four high power microscope fields with all experiments repeated at least three times. *P<0.05 compared with the control group. PL, piperlongumine.

Primers for gene amplification were from Invitrogen (Ki-67, cat. no. Mm01278617_m1; Cyclin D1, cat. no. Mm00487804_m1; MMP-9, cat. no. Mm00600163_m1; Twist, cat. no. Mm00442036_m1; Hprt1, cat. no. Mm00446968_m1). Hprt1 was used as the control. PCR amplification was performed in a 7300 RT-PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the following conditions: Initial denaturation at 94°C for 3 min; 40 cycles of denaturation at 94°C for 10 min, annealing at 55°C for 30 sec and extension at 72°C for 20 sec. Melt curve analysis was conducted from 65 to 95°C. The mixture contained 5 μ l SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc.), 1 μ l cDNA (diluted 1:50), and 2 μ l each forward and reverse primers (1 μ M) to a final volume of 20 μ l. The experiment was performed at least three times. Relative expression

values were calculated using the $2^{-\Delta\Delta Cq}$ method as previously described (21).

Statistical analysis. All experiments were performed at least three times. Values are expressed as the mean \pm standard deviation. Differences between groups were compared using analysis of variance using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference between values.

Results

PL suppresses the proliferation of GC cells. The anti-proliferative effects of PL on GC cells were determined using an MTT assay. As shown in Fig. 1, PL significantly

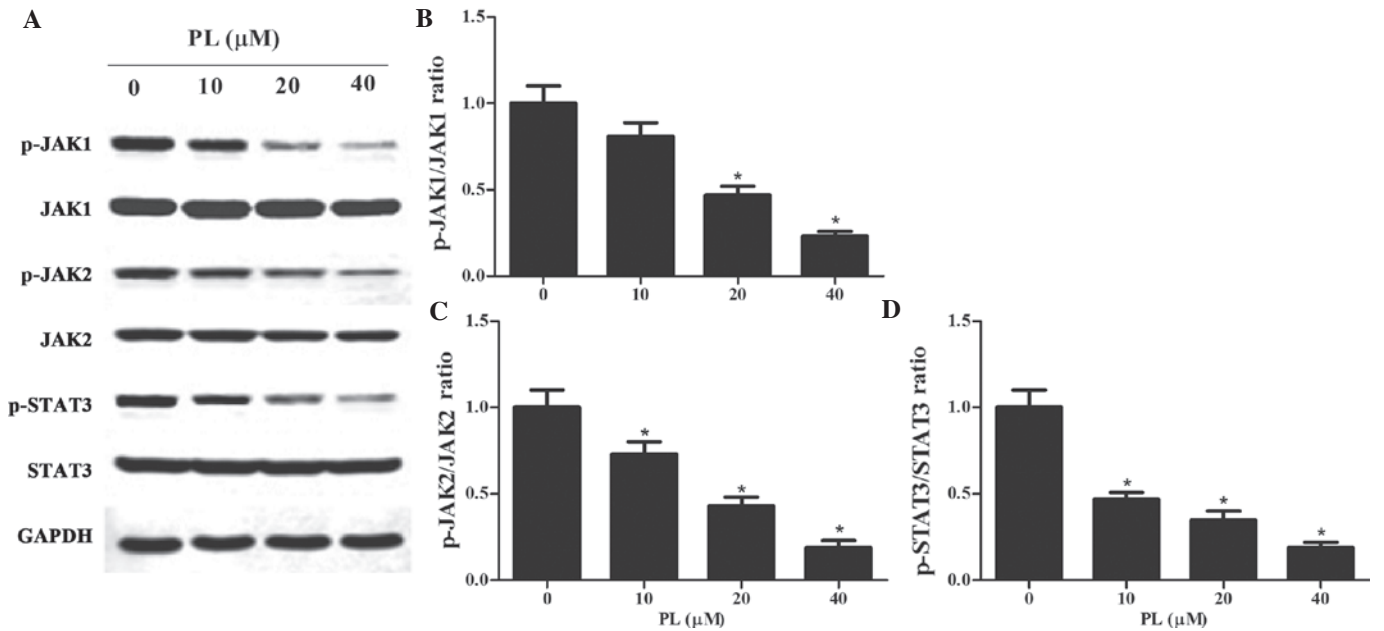


Figure 4. PL reduces STAT3 activity by downregulating JAK1/2 activity in gastric cancer cells. (A) Representative western blot of MKN45 cells treated with various concentrations of PL for 24 h. GAPDH was used as a loading control. The protein expression levels of (B) p-JAK1/JAK1, (C) p-JAK2/JAK2 and (D) p-STAT3/STAT3 were determined by densitometric analysis of the bands. *P<0.05 compared with the control group. PL, piperlongumine; p-JAK, phosphorylated Janus kinase; STAT3, signal transducer and activator of transcription 3.

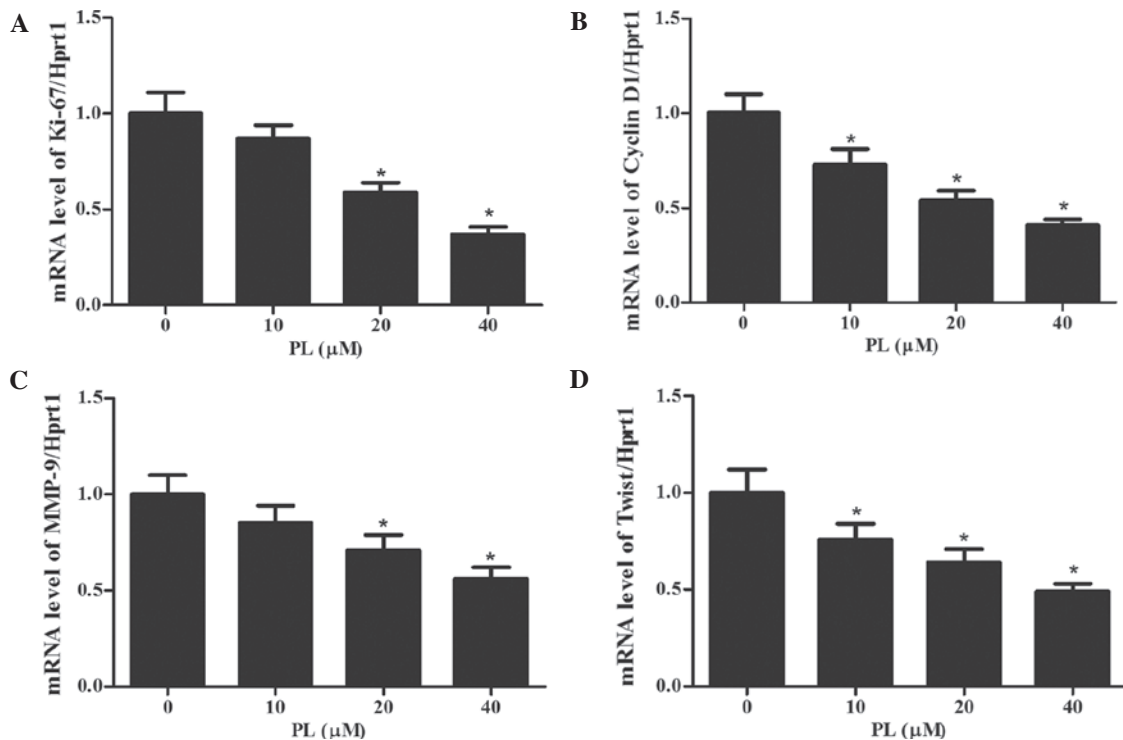


Figure 5. PL modulates the expression of STAT3-dependent tumor-associated genes. The mRNA expression levels of (A) Ki-67, (B) Cyclin D1, (C) MMP-9 and (D) Twist in MKN45 cells treated with 0, 10, 20 or 40 μM PL for 24 h were measured by reverse-transcription quantitative polymerase chain reaction analysis. Values are expressed as the mean ± standard deviation. *P<0.05 compared with the control group. PL, piperlongumine.

suppressed proliferation of MKN45 and AGS cells in a concentration- and time-dependent manner.

PL induces cell cycle arrest in G2/M phase in GC cells. To investigate whether PL inhibited the proliferation of GC cells via interfering with cell cycle progression, MKN45 and AGS

cells treated with PL for 24 h were subjected to flow cytometric cell cycle analysis. As shown in Fig. 2, an increase in the G2/M-phase population from 21 to 28, 34 and 43, along with a decrease in G0/G1 phase from 74 to 66, 58 and 53% was observed in MKN45 cells, after 24 h of treatment with PL at 0, 10, 20 or 40 μM, respectively. Similarly, an increase in

the G2/M-phase population from 21 to 33, 42 and 59%, along with a decrease in G0/G1 phase from 68 to 54, 43 and 33% was found in AGS cells after 24 h of treatment with PL at 0, 10, 20 or 40 μ M, respectively. Furthermore, slight dose-dependent increase in the number of MKN45 and AGS cells in S phase after treatment with PL was observed. All of these results suggested that PL dose-dependently induced G2/M-phase arrest in GC cells.

PL suppresses the invasion and migration of GC cells. Subsequently, the effects of PL on the invasion and migration of GC cells were assessed. Invasion of GC cells was assessed using Transwell chambers with Matrigel-coated membranes, while the membranes were kept in their original condition for the migration assay. As shown in Fig. 3A and B, PL dose-dependently inhibited the invasion and migration of MKN45 cells. Furthermore, the invasion and migration of AGS cells was also inhibited by PL in a concentration-dependent manner (Fig. 3C and D).

PL de-activates STAT3 activity by downregulating JAK1/2 activity in GC cells. STAT3 has been reported to be constitutively activated in GC cells (22,23). As activation of STAT3 is associated with the activation of upstream JAKs (24), the present study examined whether PL affected the JAK/STAT3 pathway. Western blot analysis was performed on lysates of PL-treated MKN45 cells, revealing that PL treatment downregulated p-JAK1, p-JAK2 and p-STAT3 in a concentration-dependent manner (Fig. 4). However, total protein expression of JAK1, JAK2 and STAT3 was not altered by PL treatment.

PL decreases the expression of STAT3-dependent tumor-associated genes. To investigate whether PL showed a specific effect on STAT3-regulated genes, MKN45 cells were treated with different concentrations of PL for 24 h, followed by RT-qPCR analysis of the mRNA expression of the STAT3 target genes Ki-67 (a proliferation marker), Cyclin D1 (a cell cycle regulator), MMP-9 and Twist (associated with invasion) (25-27). As shown in Fig. 5, the mRNA expression levels of all of these genes were decreased by PL in a concentration-dependent manner. These results indicated that by inhibiting STAT3, PL interferes with the expression of these STAT3-dependent genes, which may be associated with its mechanism of action.

Discussion

At present, treatments for GC have limitations due to the occurrence of relapses. Therefore, it is urgently required to develop novel treatment modalities to improve the outcome of GC therapies. The present study demonstrated the inhibitory effects of PL on GC cells and assessed the underlying mechanisms. The results showed that PL suppressed the proliferation, cell cycle progression as well as invasion and migration of GC cells through inhibition of the JAK1,2/STAT3 signaling pathway.

JAKs, comprised of JAK1, JAK2, JAK3 and TYK2, belong to a family of non-receptor tyrosine kinases (28). They are phosphorylated by cytokine and growth factor receptor signaling, which then results in activation of STAT3 (3,29-31). STAT3, a member of the STAT family of transcription factors,

exists in the cytoplasm and is tightly mediated under physiological conditions. Its activation, strictly controlled in normal tissues, contributes to tumorigenesis by driving biological processes and cellular functions, including proliferation, survival, metastasis, angiogenesis, immune evasion and inflammation (32-36). The JAK/STAT3 signaling pathway has been reported to have a central role in GC and is thus a common target of GC treatments (37,38). For example, Kim *et al* (39) demonstrated that OPB-31121 exhibited an antitumor effect on GC cells by disrupting the JAK2/STAT3 pathway. In addition, GC cell growth was reported to be reduced by suppression of the JAK2/STAT3 pathway (38,40,41). Another previous study demonstrated that JAK1/STAT3 signaling is also involved in the invasion of cancer cells (42). The present study revealed that phosphorylation of JAK1, JAK2 and STAT3 was dose-dependently inhibited by PL in GC cells. This indicated that PL may exert its effects on GC cells by suppression of JAK1 and JAK2 activation, resulting in reduced STAT3 activation.

Subsequently, the effects of PL on the expression of target genes of STAT3 relevant to proliferation (Ki-67), cell cycle progression (Cyclin D1) and invasion (MMP-9 and Twist) were assessed. The results showed that PL treatment significantly and dose-dependently reduced the mRNA expression levels of all of these genes. The present study revealed that PL suppressed the proliferation of the MKN45 and AGS cells by inducing cell cycle arrest at G2/M phase, while downregulation the mRNA expression of Cyclin D1 following incubation for 24 h. Furthermore, the present study revealed that PL inhibited the invasion of GC cells through suppressing the expression of MMP-9 and Twist, which are target genes of STAT3 relevant to cell invasion.

In conclusion, the present study was the first to reveal the inhibitory effects of PL on GC cells and to assess the underlying mechanisms. PL was demonstrated to inhibit the proliferation, cell cycle progression as well as invasion and migration of two GC cell lines. The underlying mechanisms were indicated to include the suppression of the JAK1,2/STAT3 signaling pathway as well as the inhibition of the expression of downstream genes. These results indicated that the consumption of long pepper is recommended for the prevention and treatment of GC, and that PL may represent a novel chemotherapeutic drug for GC.

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