Panax ginseng polysaccharide suppresses metastasis via modulating Twist expression in gastric cancer

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ABSTRACT

It was previously reported that an antitumor polysaccharide (PGPW1) was isolated from the root of Panax ginseng. To extend our study, we investigated here the anti-invasive and metastatic effects of PGPW1 on human gastric cancer cell line HGC-27 and tried to determine its possible mechanism of action. Both scratch wound-healing and Transwell assay identified that PGPW1 dose-dependently inhibited migration and invasiveness of HGC-27 cells. Furthermore, results of western blot showed that protein levels of Twist and AKR1C2 were inhibited by PGPW1, whereas an increase of NF1 was observed. Moreover, down-regulation of Twist expression by PGPW1 blocked epithelial–mesenchymal transition (EMT), characterized by a gain of epithelial cell markers, E-cadherin, and loss of the mesenchymal markers, vimentin and N-cadherin, at protein levels. Collectively, we confirmed that PGPW1 decreased migration and invasion of HGC-27 cells by regulation of Twist, AKR1C2, NF1, E-cadherin, vimentin and N-cadherin expression. In conclusion, PGPW1 may serve as a powerful chemopreventive agent against gastric cancer metastasis.

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1. Introduction

Gastric cancer is one of the most common malignant cancers with poor prognoses and is one of the leading causes of cancer mortality worldwide [1]. Current therapies for gastric cancer mainly consist of chemotherapy, surgery and radiation [2,3]. However, these conventional strategies for treatment of human gastric cancer are not yet satisfactory in many cases [4]. One important cause of the failure in anti-cancer therapies is the development of tumor metastasis. Most cancer patients do not die because of the primary cancer, but because of the metastatic cancer [5]. Metastasis of gastric cancer is not only a sign of deterioration, but also the major cause of treatment failure and death. It is necessary to discover more effective drugs to improve the life expectancy of gastric cancer patients. In our previous work [6], we found that the invasive potential of HGC-27 was substantially reduced by Twist depletion, which was accompanied by dramatically morphologic changes from scattered and fibroblast-like shapes to tightly packed cobblestone, flat cell body and smaller cell volume morphology. In addition, up-regulation of epithelial markers such as E-cadherin and down-regulation of mesenchymal markers such as vimentin and fibronectin were also observed in Twist-depleted HGC-27 cells. Hence, both morphologic and molecular changes indicated that Twist depletion resulted in abnormal epithelial–mesenchymal transition (EMT), thus leading to the progression of cancer into an invasive form. We also used microarray analysis to examine the gene expression profiles in Twist-depleted HGC-27 cells [7]. The results suggested that NF1 and AKR1C2 were major downstream targets of Twist, which might contribute to tumor invasion and metastasis. Therefore, searching for a novel drug that could modulate the expression of Twist, AKR1C2 and NF1 is important for gastric cancer therapy.

Recently, there has been increasing interest in the screening of bioactive polysaccharides from natural sources because of their broad spectrum of therapeutic properties, relatively low toxicity [8]. Some of plant polysaccharides have exhibited strong antitumor properties and can be explored as novel potential antitumor agents [9–14]. In a former study [15], we isolated a homogeneous
polysaccharide (PGPW1) from the root of *Panax ginseng*. Its molecular weight was estimated to be $3.5 \times 10^5$ Da by high performance liquid chromatography (HPLC) and gas chromatography (GC) analysis identified that PGPW1 contained Glc, Gal, Man and Ara in the molar ratio of 3.3:1.2:0.5:1.1. Preliminary studies have indicated that it exhibited inhibitory action on tumor cell migration and invasion of human bladder T24 cells in vitro. However there is no information published about the anti-metastasis effect and possible action mechanism of PGPW1 on human gastric cancer. In view of this situation, the primary objective of this study was to evaluate the anti-metastasis effects of PGPW1 using human gastric cancer HGC-27 cells and to ascertain whether Twist and down-stream factors were the target gene of this polysaccharide to prevent tumor from invasiveness and metastasis. The correlation between Twist and EMT in HGC-27 cells was also examined.

2. Materials and methods

2.1. Materials and chemicals

*P. ginseng* was purchased from the local drugstore in Beijing city of China. Sepharose 6 Fast Flow and DEAE Sepharose Fast Flow were purchased from Amersham Pharmacia Biotech Inc.

2.2. Extraction, isolation and purification of the polysaccharide PGPW1

PGPW1 was extracted from *P. ginseng* with a method as previously described [15]. Briefly, the dried roots of *P. ginseng* were refluxed with 95% ethanol at 90 °C for 2 h under reflux to remove lipids. Subsequently, the dried ethanol-extracted residue was extracted with distilled water at 100 °C for 3 h, filtered through gauze and centrifuged to remove water-insoluble materials. The aqueous extract was concentrated, and then mixed with four volumes of cold 95% ethanol at 4 °C for 24 h to isolate the polysaccharides. The precipitate was collected by centrifugation and was deproteinated three times with mixed reagent (CHCl₃:n-ButOH = 4:1, v/v) as described by Sevag method [16]. Finally the supernatant was washed with anhydrous ethanol, acetone and ether in turn, and then dried to yield the crude polysaccharides (CPGPW).

The CPGPW were purified on a DEAE Sepharose Fast Flow column (40 × 2.6 cm) eluted with distilled water and different concentrations of stepwise NaCl solution (0.15, 0.3, 0.6 and 2.0 M NaCl) at a flow rate of 4.0 ml/min. Total carbohydrate content of each tube was measured at 490 nm by the phenol-sulfuric acid colorimetric method [17]. The fractions eluted by distilled water were further fractioned on a Sepharose 6 Fast Flow column (90 × 2 cm) and eluted with 0.15 M NaCl at a flow rate of 2 ml/min. The main polysaccharide fraction was collected and lyophilized to obtain white purified polysaccharide (PGPW1) and used for further study.

2.3. Cell lines and cell culture

HGC-27 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at in a 5% CO₂ humidified incubator at 37 °C.

2.4. Plasmid construction and transfection

pSUPER RNAi system (Oligoengine) was used to construct Twist silencing vectors and Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection according to the manufacturer’s instructions, and the detailed methods for making shRNA-based stable knockdown of Twist cell lines was described previously [6]. The resulting cell colonies whose Twist expression was most intensively repressed by RNAi fragment were assigned as ShTwist and subjected to further experiments.

2.5. Scratch wound-healing assay

The cell migration assay was performed as previously described [18]. Briefly, HGC-27 cells were allowed to grow into full confluence in 24-well plates at a concentration of 5 × 10⁵ cells per well. After 24 h incubation, a uniform scratch was made down the center of the well using a sterile plastic pipette tip, followed by washing twice with PBS. Vehicle control and various concentrations of PGPW1 (50, 100 and 200 μg/ml) were added to the respective wells for 24 h. Meanwhile, an equal amount of Twist-depleted HGC-27 cells served as positive control. Photographic imaging was performed using the Nikon inverted microscope. The migration distances between the leading edge of the migrating cells and the edge of the wound were compared. The migration rate was quantified as the following equation: migration rate = [(migration distance of PGPW1-treated or Twist-depleted cells/migration distance of untreated control cells) × 100%]. Experiments were independently performed at least three times. The migration rate of the control was set as 100%.

2.6. In vitro invasion and migration assays

The invasion assay was performed as previously described [19]. Using a Transwell cell culture chamber with filter of 8 μm pore size coated with Matrigel on the upper surface. Briefly, HGC-27 cells were seeded in the upper chamber at a density of 2 × 10⁴ cells/well in 200 μl of serum-free medium and the lower chamber was filled with complete pretreatment medium containing 10% FBS. PGPW1 (0, 50, 100 and 200 μg/ml) were put into both the chambers respectively. After 24 h of incubation at 37 °C, the nonmigrated cells on the upper surface of the Transwell membrane were completely wiped off with a cotton swab. The cells that penetrated to the lower surface of the Transwell membrane were counted under a light microscope. A blinded viewer counted the number of cells present in five fields per chamber. The migration rate was calculated from the following formula: the percentage of migration rate = [(migration cellsnumber/migration cellscontrol) × 100%]. Twist-depleted HGC-27 cells were performed in the same way and served as positive control.

2.7. Western blot analysis

HGC-27 cells were seeded in T-75 tissue culture flasks at a concentration of 1.8 × 10⁵ cells/ml and incubated with 50, 100 and 200 μg/ml of PGPW1. After 24 h incubation, the cells were harvested and lysed, and protein concentrations were quantified using the BioRad protein assay as described in manufacturer’s manual. Equal amounts of proteins from the extracts was electrophoresed on 10% SDS-PAGE gel and subsequently transferred onto nitrocellulose membranes. The membranes were blocked with 5% skimmed milk for 2 h at room temperature and incubated with the primary antibodies Twist (1:200), N-cadherin (1:500), E-cadherin (1:500), β-actin (1:2000) overnight at 4 °C, followed by a conjugated secondary antibody to peroxidase.

2.8. Statistical analysis

The data were expressed as the means ± SD. The significance of differences between values was checked with one-way ANOVA. A level of $P < 0.05$ was accepted as statistical significance.
3. Results and Discussion

3.1. PGPW1 inhibited cell migration and invasion in HGC-27 cells

Since invasion and migration are common features in the process of tumor metastasis, a wound healing assay and a Transwell assay are used to investigate the influence of PGPW1 on the invasive and migratory abilities of HGC-27 cells in vitro.

To explore the effect of PGPW1 on the motility ability of HGC-27 cells, the scratch wound assay was performed. After 24 h wound healing, a wound in HGC-27 cells was able to move to an almost complete closure, while cells treated with PGPW1 (50, 100, and 200 μg/ml) or transfected with shTwist still showed a noticeable wound at the same time point (Fig. 1). This suggests that PGPW1 inhibits the cell motility ability in a human gastric cancer cell line HGC-27, comparable to Twist shRNA treatment.

One important characteristic of metastasis is the migratory ability of tumor cells. Next, we also assessed the migratory ability of HGC-27 cells response to PGPW1 treatment. As shown in Fig. 2, compared to vehicle-treated cells, PGPW1 treatment significantly dose-dependently attenuated the invasion of HGC-27 cells in the range of 50-200 μg/ml. Especially at the concentration of 200 μg/ml, the percentage of migration rate was close to the level of Twist-depleted control cells, suggesting that suppressing effect of PGPW1 on cell migration was comparable to Twist shRNA treatment to HGC-27 cells.

3.2. PGPW1 inhibited Twist and AKR1C2, but up-regulate NF1 expressions in at protein levels in HGC-27 cells

Because Twist and its two downstream genes NF1 and AKR1C2 have been implicated in cell survival, proliferation and metastasis [6], we examined the effect of PGPW1 on the expressions of these genes at protein levels in HGC-27 cells using western blot analysis. PGPW1 suppressed the protein expression of Twist in a concentration-dependent manner. Similar responses were observed for its downstream target AKR1C2, whereas the

downstream proteins of NF1 were drastically increased by PGPW1. In addition, Twist knockdown in HGC-27 cells leads to a decrease of AKR1C2 and an increase of NF1, thus leading to the attenuation of metastatic potential of the gastric cancer cell line. Our data indicated that suppression of the Twist by PGPW1 could partially affect its two downstream proteins NF1 and AKR1C2 in HGC-27 cells (Fig. 3).

3.3. PGPW1 inhibited vimentin, but up-regulate E-cadherin expressions at protein levels in HGC-27 cells

It has been reported that epithelial-mesenchymal transition (EMT), morphologic alteration from epithelial type to mesenchymal phenotype, is one of the major events during tumor invasion and metastasis [20]. This process is often accompanied by the dissolution of epithelial tight junctions, loss of cell adhesion, down-regulated expression of epithelial markers, such as E-cadherin, α-catenin, and β-catenin, but acquired expression of mesenchymal
work also presents the underlying molecular events occurring in
presence of PGPW1. Following exposure to PGPW1, Twist and
AKR1C2 protein levels in HGC-27 cells were decreased, whereas
an increase of NF1 protein expression was observed. At the same
time, PGPW1 treatment retarded EMT progression in HGC-27 cells,
with a gain of epithelial cell markers, E-cadherin, and loss of the
mesenchymal markers, vimentin and N-cadherin. These results
again strengthened the fact that Twist was essential in EMT
during tumor invasion and migration in HGC-27 cells and PGPW1 may
be useful as a potent agent to prevent tumor metastasis for gastric
cancer.

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