

Panax ginseng polysaccharide induces apoptosis by targeting Twist/AKR1C2/NF-1 pathway in human gastric cancer

Cong Li^a, Zhen-Nan Tian^a, Jian-Ping Cai^a, Ke-Xin Chen^a, Bao Zhang^c, Mei-Yan Feng^a, Qing-Tao Shi^a, Rui Li^c, Yu Qin^a, Jing-Shu Geng^{a,b,*}

^a Department of Pathology, the Third Affiliated (Tumour) Hospital, Harbin Medical University, Harbin 150040, China

^b Department of Medical Genetics, Harbin Medical University, Harbin 150086, China

^c Department of Urological Surgery, Aerospace Central Hospital (Aerospace Clinical Medical College of Peking University, Peking University), Beijing 100049, China

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ABSTRACT

In the present study, we isolated and screened an antitumor polysaccharide (PGP2a) from the roots of *Panax ginseng*. Chemical composition analysis indicated PGP2a was an acidic protein–polysaccharide. The average molecular weight was estimated to be 3.2×10^4 Da. According to gas chromatography (GC) result, PGP2a consisted of galactose, arabinose, glucose and galacturonic acid in the molar ratio of 3.7:1.6:0.5:5.4, respectively. MTT assay showed that PGP2a had a potent inhibitory effect on the growth of HGC-27 cells in a dose-dependent fashion. Furthermore, the number of HGC-27 cells arrested in G2/M phase, and the percentage of apoptotic cells were increased in response to PGP2a treatment along with concentration increasing. Moreover, western blotting analysis showed that protein expressions of Twist and AKR1C2 were suppressed by PGP2a, whereas an increase of NF1 was observed at protein level. Taken together, these findings suggested that PGP2a could be developed as a novel antitumor agent acting on Twist related gene for human gastric cancer therapy.

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1. Introduction

Gastric cancer is one of the most common cancers that originates from the epithelium gastric carcinoma and is a lethal cancer in the world (Sun et al., 2011). Currently, the major conventional cancer therapies for this malignancy include surgery, chemotherapy and radiotherapy (Rugge, 2007). In spite of the progress made in the treatment of gastric cancer, these therapies have numerous limitations due to low response and poor survival rate (Liu, Zhu, Chang, & Hang, 2012), indicating that there is an urgent need for the development of novel therapeutic strategies.

A growing body of evidence indicates that Twist is a potential oncogene. Over-expression of Twist may contribute to various properties of tumor cells, such as inhibition of differentiation of tumor, induction of tumorigenesis (Maestro et al., 1999), promotion of invasion and metastasis (Lee et al., 2006; Martin, Goyal, Watkins, & Jiang, 2005; Song et al., 2006; Yang et al., 2004),

enhancement of drug resistance (Li, Wood, Becker, Weeraratna, & Morin, 2006; Wang et al., 2004), stimulation of angiogenesis (Mironchik et al., 2005) and induction of genomic instability (Vesuna, Winnard, Glackin, & Raman, 2006). In our previous study, we studied the functions of Twist on regulating migration rate, apoptosis, and gene expression in gastric cancer cells. Twist knockdown by RNAi approach in Twist-high-expressed gastric cancer HGC-27 cells resulted in suppressing migration ability, increasing induction of apoptosis in response to arsenic oxide and elevating cell cycle arrest (Feng, Wang, Shi, Yu, & Geng, 2009). To further investigate the potential targets and interacting genes of Twist in human gastric cancer, we performed microarray analysis to examine the gene expression profiles in Twist-depleted HGC-27 cells. Our results showed that NF1, RAP1A, SRPX, RBL2, PFDN4, ILK, F2R, ERBB3 and MYB were up-regulated, whereas AKR1C2, FOS, GDF15, NR2F1, ATM and CTIP5 were down-regulated after Twist depletion (Feng, Wang, Song, et al., 2009). These results provided evidence that Twist regulated the expression of several genes involved in the differentiation, adhesion and proliferation of gastric cancer cells. Among them, NF1 and AKR1C2 expressions varied significantly in HGC-27 cells before and after Twist knockdown by RNAi. Collectively, these data suggest that NF1 and AKR1C2 are major downstream targets of Twist. Both NF1 and AKR1C2 genes could induce tumor progression, promote cell growth and drive

* Corresponding author at: Department of Pathology, the Third Affiliated (Tumour) Hospital of Harbin Medical University, Harbin 150040, China. Tel.: +86 45186298232.

E-mail addresses: jingshugeng@126.com, jingshu.geng@hotmail.com (J.-S. Geng).

oncogenesis in HGC-27 cells. Thus, Twist and its two downstream genes (AKR1C2 and NF1) may represent promising molecular targets for gastric cancer therapy.

Recently, the application of natural compounds to prolong the life of patients with gastric cancer has increased in popularity (Xu et al., 2003). *Panax ginseng* C.A. Meyer (*P. ginseng*) is a well-known traditional Chinese medicine, and has been used for several thousand years with mysterious powers in the Orient as a tonic, prophylactic and restorative agent (Sun, 2011). *P. ginseng* contains many active components including ginsenosides, essential oil, peptidoglycans, polysaccharides, nitrogen-containing compounds, fatty acids and phenolic compounds (Choi, 2008; Lee et al., 2010; Sun, 2011; Xiang, Shang, Gao, & Zhang, 2008). It has been reported that *P. ginseng* polysaccharides have immunomodulation, anti-tumor, anti-ulcer, anti-radiation, antioxidant, hypoglycemic activities, and so on (Sun, 2011). In our research laboratory, we isolated different polysaccharide fractions from *P. ginseng* and preliminarily examined their inhibitory effect on human gastric cancer HGC-27 cells. The MTT assay showed that one acidic polysaccharide possessed potent suppressing effect on the proliferation of HGC-27 cells. Despite the emerging evidence of its importance, no information is available regarding the anticancer effects of *P. ginseng* polysaccharides on human gastric cancer cells, and its underlying mechanism of action is still largely unknown. Therefore, we used human gastric cancer HGC-27 cells to investigate whether treatment with this polysaccharide could induce cell apoptosis and modulates the expression of Twist, AKR1C2 and NF1 in HGC-27 cells.

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 was purchased from Amer sham (Sweden). DEAE Sepharose Fast Flow was purchased from Pharmacia Biotech.

2.2. Isolation and purification of the polysaccharide

The dried roots of *P. ginseng* were refluxed with 95% ethanol at 90 °C for 2 h under reflux to remove small lipophilic and pigment molecules. 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 deproteinated by Sevag method (Staub, 1965). Finally the supernatant was washed with anhydrous ethanol, acetone and ether in turn, and then dried to yield the crude polysaccharides (CPGP).

The CPGP was dissolved in distilled water, centrifuged, and then the supernatant was applied to a column of DEAE Sepharose Fast Flow equilibrated with 0.9% NaCl. After loading with sample, the column was first eluted with 1.5 column volume of distilled water, and then eluted with the same volume of step gradient concentrations of NaCl aqueous solution (0.15, 0.3, 0.6 and 2 M NaCl) at 4 mL/min. Test tubes were collected by an automated step-by-step fraction collector. Guided by the calorimetric total carbohydrate test using the phenol–sulfuric acid method, five main fractions were combined and named as PGPW, PGP1, PGP2, PGP3 and PGP4, respectively. These polysaccharides were further purified by size-exclusion chromatography on a Sepharose 6 Fast Flow column (90 cm × 2 cm) eluted with 0.15 M NaCl at a flow rate of 2 mL/min to afford respective major purified polysaccharide.

2.3. Analysis of physico-chemical characteristics of the polysaccharide

Total carbohydrate contents in purified samples were determined according to the phenol–sulfuric acid method using glucose as a standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid contents were measured by the carbazole–sulfuric acid method using glucuronic acid as standard (Blumenkrantz & Asboe-Hansen, 1973). Protein contents were quantified according to the Bradford's method using bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.4. Molecular weight determination and monosaccharide composition analysis of the polysaccharide

The homogeneity and average molecular weight of PGP2a were identified by high performance gel permeation chromatography (HPGPC) (Jahanbin, Gohari, Moini, Emam-Djomeh, & Masi, 2011). The sample solution was applied to an Agilent 1100 system equipped with a TSK-GEL G4000PWXL column and an evaporative light scattering detector (ELSD), eluted with 0.7% Na₂SO₄ as the mobile phase at a flow rate of 0.8 mL/min. The molecular weight was estimated by reference to the calibration curve made from a Dextran T-series standard of known molecular weight (Mw 2000, 1000, 400, 70, and 10 kDa).

Gas chromatography (GC) analysis was used for identification and quantification of the monosaccharides. PGP2a (10 mg) was hydrolyzed with 2 M TFA at 110 °C for 3 h (Zhang, Xiao, He, & Sun, 2011). The monosaccharide was conventionally converted into the alditol acetate as described previously (Wu, Cui, Zhang, & Li, 2012; Wu, Zhang, Lang, et al., 2012) and was analyzed by GC, which was performed on a Vavian 3400 instrument (Hewlett-Packard Component, USA) equipped with SE-30 column (30 m × 0.25 mm × 0.25 μm) and detected with a flame ionization detector (260 °C). The column temperature was maintained at 120 °C for 2 min, and then increased to 250 °C for 2 min at a rate of 8 °C/min.

2.5. Cell lines and cell culture

HGC-27 (gastric carcinoma cells) and AGS (human normal gastric epithelial cells) were purchased 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. The cells were incubated in an atmosphere of 5% CO₂ at 37 °C and sub-cultured every 3 days.

2.6. 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 (Feng, Wang, Song, et al., 2009). The detailed methods for making shRNA-based stable knockdown of Twist cell lines was described previously (Feng, Wang, Song, et al., 2009). The resulting cell colonies whose Twist expression was most intensively repressed by RNAi were assigned as ShTWSIT and subjected to further experiments.

2.7. Antitumor components screening assay

HGC-27 cells (1 × 10⁵ cells per well) were seeded in a 96-well plate with the varying concentrations of PGPw, PGP1, PGP2, PGP3 and PGP4 (25, 50, 100, 200, 400 and 800 μg/mL), respectively.

After incubation at 37 °C for 24 h, MTT was dissolved in phosphate-buffered saline (PBS) at 5 mg/mL and added to culture media at a final concentration of 0.5 mg/mL. After 4 h, media were removed, and 100 µL DMSO was added to each well to dissolve purple crystals of formazan. Absorbance was measured by a multi-detection microplate reader (BIO-RAD, USA) at 570 nm. Cell growth inhibition rate (%) was calculated using the following equation: Inhibitory rate (%) = $(1 - A_{\text{treatment}}/A_{\text{control}}) \times 100\%$. The 50% inhibitory concentration (IC₅₀) value was determined as the concentration that caused 50% inhibition of cell proliferation. All experiments were performed in triplicate.

2.8. Measurement of cell viability after PGP2a treatment for 24, 48 and 72 h

HGC-27 or AGS cells (1×10^5 cells per well) were seeded in a 96-well plate with the varying concentrations of PGP2a (25, 50, 100, 200 and 400 µg/mL). After incubation at 37 °C for 24, 48 and 72 h, the cell viability was analyzed by MTT method as described above. The given values were calculated from the mean of three different experiments. The cell viability was expressed as the optical density ratio of the treatment to control.

2.9. Apoptosis assay

Flow cytometric analysis was performed to identify and quantify the apoptotic cells using Annexin V and PI staining, as described previously. In brief, the HGC-27 cells were seeded in 6-well plates at a density of 1×10^5 cells/well. After treatment with PGP2a at concentrations of 100, 200 and 400 µg/mL for 48 h, both adherent and floating cell populations were collected by brief trypsinization and rinsed with phosphate buffered saline (PBS) twice, then subjected to Annexin V and PI staining using an Annexin V-FITC/PI apoptosis detection kit (BD Bioscience) following the step-by-step protocol provided by the manufacturer. After staining, the quantification of apoptotic cells was measured with a Becton-Dickinson FACS-Calibur flow cytometer.

2.10. Morphology observation

AO/EB fluorescent staining method was used to observe apoptotic morphology of individual cells in a cell population as described previously (Tian et al., 2008). Briefly, HGC-27 cells were treated with vehicle or PGP2a at 400 µg/mL for 48 h, and then photographs were taken under an inverted fluorescence microscope (Nikon TE2000, Tokyo, Japan) after AO/EB staining.

2.11. Cell cycle assay

Cell cycle distribution was studied by flow cytometry analysis. Briefly, HGC-27 cells were treated with PGP2a at different concentrations (100, 200 and 400 µg/mL) for 48 h. After drug treatment, cells were trypsinized, washed with PBS and fixed in 70% cold ethanol at 4 °C overnight. Thereafter, cells were washed, and then stained with 20 µg/mL of PI and 50 µg/mL of RNase A at room temperature for 30 min. The cell cycle distribution of 10,000 cells was recorded by a Becton-Dickinson FACS-Calibur flow cytometer and the percentage of cells at G0/G1, S and G2/M phases were analyzed with ModFit LT V3.0 software.

2.12. Western blot analysis

Western blot was performed for detection of Twist, NF1, AKR1C2, Bid, Bax, Bcl-2, Bcl-xl, PARP, cleaved PARP, Fas, Fas ligand, Capase 3, Caspase 8 and 9 proteins using 10% SDS/PAGE (Li et al.,

Table 1

IC₅₀ values on inhibitory growth of HGC-27 cells by five *P. ginseng* polysaccharides fractions eluted by DEAE sepharose fast flow column.

Fractions	IC ₅₀ (µg/mL)
PGPW	255.742
PGP1	400.21
PGP2	115.71
PGP3	302.04
PGP4	250.17

2012). Briefly, proteins were electrotransferred onto a nitrocellulose filter. After blocking in blocking buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) containing 5% milk for 1 h, the membranes were incubated over night with specified primary antibody, followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma, St. Louis, MO, USA). The antibodies used included Twist, NF1, AKR1C2, Bid, Bax, Bcl-2, Bcl-xl, PARP, cleaved PARP, Fas, Fas ligand, Capase 3, Caspase 8 and 9 antibodies (Cell Signaling Technology, Beverly, MA, USA). β-Actin level was used as a control.

2.13. Statistical analysis

All data were expressed as means ± S.D. The means of the different groups were compared by one-way ANOVA test. All statistical analyses were performed with the SPSS13.0 software (SPSS, Chicago, IL, USA). Significant differences were defined as $P < 0.05$.

3. Results and discussion

3.1. Isolation and purification

Crude polysaccharides were extracted from defatted roots of *P. ginseng* with distilled water and precipitated by the addition of 4 volumes of 95% ethanol. Following deproteinization by the Sevag method, a crude polysaccharide fraction, referred to as CPGP, was obtained with a yield of 9.2% (w/w). Then the crude polysaccharides CPGP were purified by DEAE Sepharose Fast Flow column, and five fractions (PGPW, PGP1, PGP2, PGP3 and PGP4) were eluted with water and 0.15, 0.3, 0.6 and 2 M NaCl aqueous solutions, respectively. Five polysaccharide fractions were pooled, dialyzed, lyophilized and were further determined to their anti-proliferation effect on human gastric cancer HGC-27 cells by MTT assay. The results demonstrated that the fraction PGP2 exhibited the maximum inhibitory effect against the proliferation of HGC-27 cells (Table 1). Therefore, we loaded PGP2 on a Sepharose 6 Fast Flow column eluting with 0.15 M NaCl buffer to further purify this fraction. By this process, one purified polysaccharide (PGP2a) was obtained and used for subsequent study of chemistry and biological activities.

3.2. Chemical characterization of the polysaccharide

The total carbohydrate content, uronic acid and protein analyses showed that crude extract PGP2a was an acidic polysaccharide-protein complex containing 75.8% of total carbohydrate, 42.3% of uronic acid and 9.9% of protein. HPGC indicated that PGP2a was a homogeneous and purified polysaccharide. Its average molecular weight was determined to be 3.2×10^4 Da in reference to standard dextrans (Fig. 1). According to retention time of the alditol acetate derivatives in GC, PGP2a was composed mainly of galactose, arabinose, glucose and galacturonic acid in the molar ratio of 3.7:1.6:0.5:5.4.

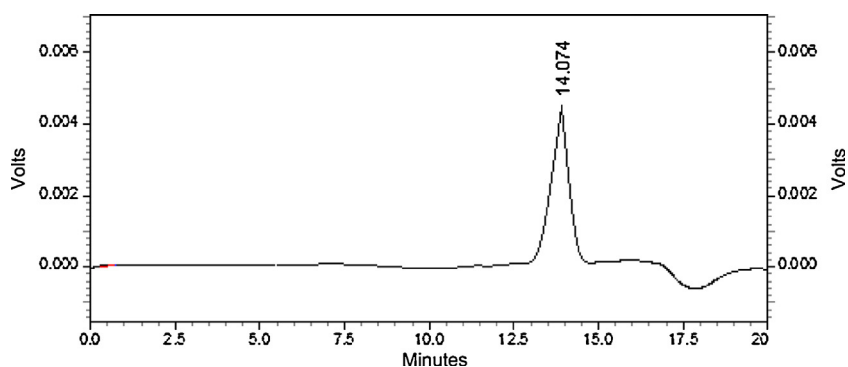


Fig. 1. HPGPC profile of PGP2a.

3.3. Effect of PGP2a on HGC-27 cell proliferation

To investigate the effect of cytotoxic activity of PGP2a on cell viability, HGC-27 cells were treated with various concentrations of PGP2a (25, 50, 100, 200 and 400 $\mu\text{g}/\text{mL}$) for 24, 48 and 72 h. After treatment, percent survival was determined as compared to untreated cells. The results show that PGP2a markedly inhibited the cells proliferation in a concentration-dependent manner, and the effects were more significant at 48 h (Fig. 2A). Therefore, we chose the concentration from 100 to 400 $\mu\text{g}/\text{mL}$ and 48 h treatment time for the following study. In addition, PGP2a weakly affected the proliferation of human normal gastric epithelial AGS cells (Fig. 2B), indicating it has no toxicity on normal cells.

3.4. Effect of PGP2a on HGC-27 cell apoptosis and cell cycle

To determine whether the programmed apoptosis involved in tumor regression by PGP2a treatment, morphological observation and annexinV-FITC/PI double staining of HGC-27 cells were examined. We treated HGC-27 cells with 400 $\mu\text{g}/\text{mL}$ PGP2a for 48 h, and the apoptotic morphology of individual HGC-27 cells in the cell population was observed by AO/EB staining (Fig. 3A).

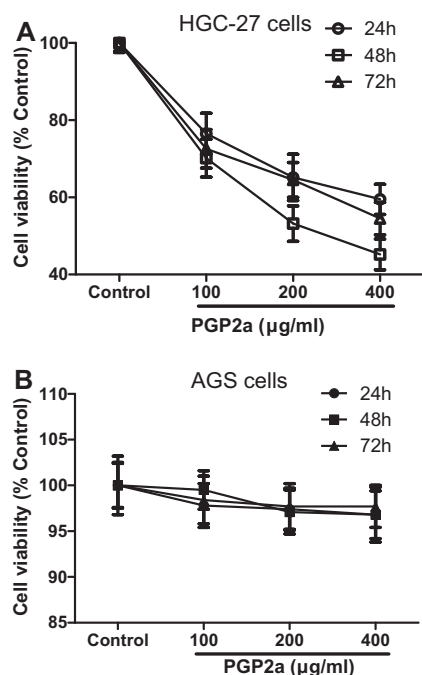


Fig. 2. Effect of PGP2a on the proliferation of (A) HGC-27 and (B) AGS cells. Each data indicated the mean $\pm$ S.D. of three independent experiments.

Normal nuclear morphology was observed in untreated cells. In contrast, PGP2a-treated cells demonstrated typical apoptotic morphology, such as chromatin aggregation, nuclei contraction and red or yellow-red nuclei, due to the loss of their membrane integrity. Similar apoptotic morphology was observed in cells interfered with Twist shRNA.

To quantify apoptosis in HGC-27 cells triggered by PGP2a, cells were stained with Annexin V-FITC/PI, and then subsequently analyzed by flow cytometry. The percentage of apoptotic cells was expressed as Annexin V positive cells. As shown in Fig. 3B, treatment of HGC-27 cells with PGP2a in concentration of 0, 100, 200 and 400 $\mu\text{g}/\text{mL}$ for 48 h resulted in a dose-dependent increase in the number of total apoptotic cells, from 6.6% to 21.7%, 42.1% and 65.0%, respectively. Furthermore, treatment with Twist shRNA in HGC-27 cells also induced a significant percentage of apoptotic cells. These results suggested that the reduction in the viability of the HGC-27 cells was at least partly attributable to apoptosis induced by PGP2a.

The cytotoxicity caused by PGP2a may be in part due to anti-proliferative and proapoptotic effects. The effect of PGP2a on cell cycle progression was analyzed by flow cytometry. As shown in Fig. 3C, exposure to 100–400 $\mu\text{g}/\text{mL}$ PGP2a caused an increase of the G2/M phase population from 14.7% to 46.9%, as compared to 6.0% of G2/M phase cells in untreated control samples. An evident increase of G2/M phase cells was also detected in Twist-knockdown HGC-27 cells. Hence, PGP2a exerted growth-inhibitory effects via G2/M phase arrest in a concentration-dependent manner.

3.5. Effect of PGP2a on activation of Bcl-2 family proteins

To investigate whether the mitochondrial apoptotic events involved in PGP2a-induced apoptosis, we first analyzed the changes in the levels of the Bcl-2 family proteins. Following the treatment of HGC-27 cells with PGP2a, we observed that PGP2a treatment resulted in a significant increase of Bax expression, and a decrease of Bcl-2 expression, which led to an increase in the ratio of Bax/Bcl-2 (Fig. 4). In addition, PGP2a also decreased the expression of Bcl-xL. All three Bcl-2 family proteins behaved in the same trend in cancer cells treated with Twist ShRNA. These results suggested that changes in the ratio of the proapoptotic and antiapoptotic Bcl-2 family proteins might contribute to PGP2a-promoted apoptosis.

3.6. Effect of PGP2a on activation of caspase-3, caspase-9 and PARP

To further study the mechanism of PGP2a-induced apoptosis in HGC-27 cells, we investigated the involvement of the mitochondrial-mediated intrinsic apoptotic pathway by assessing the cleavage of caspases 3, 9 and PARP by Western blot analysis. Compared with the untreated control, the expressions of the active form of caspase-3, caspase-9 and cleaved PARP increased

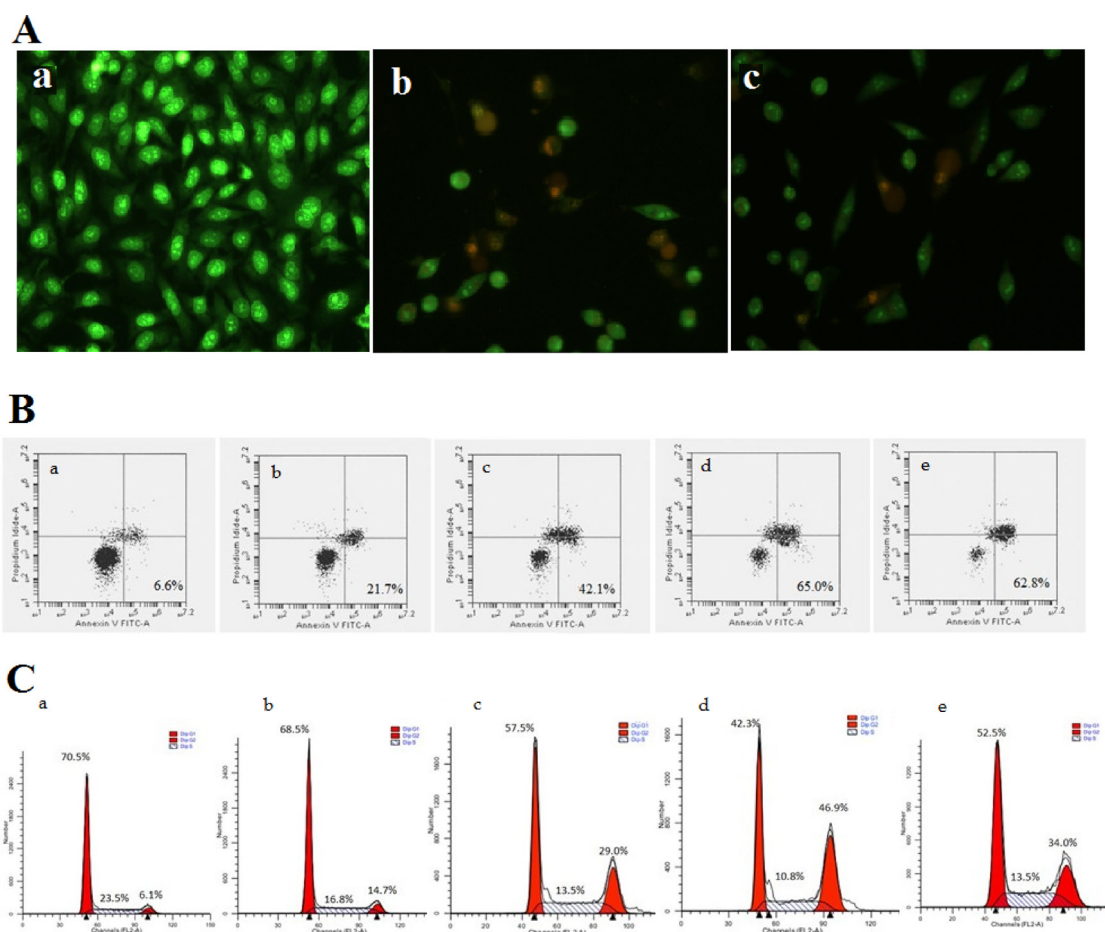


Fig. 3. Apoptosis induced by PGP2a in HGC-27 cells. (A) Morphological changes in HGC-27 cells after exposure to PGP2a for 48 h using AO/EB staining under an inverted fluorescence microscope (200 $\times$). The arrows indicate the cells undergoing apoptosis. (a) Control, (b) PGP2a (400 μ g/mL), (c) Twist-shRNA HGC-27 cells. (B) PGP2a-induced apoptosis in HGC-27 cells using annexin V-FITC/PI double staining. (a) Control, (b) PGP2a (100 μ g/mL), (c) PGP2a (200 μ g/mL), (d) PGP2a (400 μ g/mL), (e) Twist-shRNA HGC-27 cells. (C) Cell cycle distribution of HGC-27 cells after treatment with different concentrations of PGP2a for 48 h. (a) Control, (b) PGP2a (100 μ g/mL), (c) PGP2a (200 μ g/mL), (d) PGP2a (400 μ g/mL) and (e) Twist-shRNA HGC-27 cells.

significantly after HGC-27 cells were treated with PGP2a (Fig. 5). Similar trend was also observed in Twist-depleted HGC-27 cells. Collectively, these results indicated that mitochondria-mediated apoptotic pathway might be involved in PGP2a induced apoptosis in HGC-27 cells.

3.7. Effect of PGP2a on activation of caspase-8, Bid, Fas and Fas ligand

Extrinsic pathway, namely death receptor-mediated pathways, is the interaction of the cell surface receptors with their ligands to

activate the downstream effectors. For example, The engagement of Fas and Fas ligand on the cell surface induces the activation of caspase-8, followed by the cleavage of a pro-apoptotic Bcl-2 family member, Bid, to its truncated form (tBid). As a result, tBid translocates to mitochondria which leads to mitochondria damage and amplifies apoptotic signals by activating the mitochondrial pathway (Esques, Desagher, Antonsson, & Martinou, 2000; Wei et al., 2000).

To evaluate whether PGP2a induced apoptosis via receptor-mediated pathway, HGC-27 cells were treated with PGP2a (100, 200 and 400 μ g/mL) to detect the expression of Fas, Fas ligand, pro-caspase 8, and Bid by Western blot analysis. As shown in

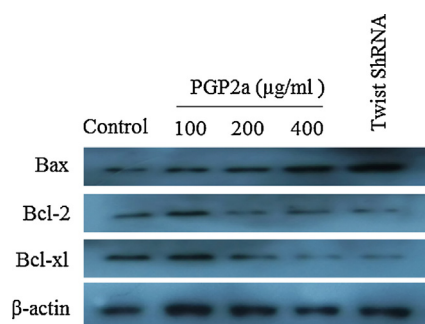


Fig. 4. Effect of PGP2a on Bcl-2 family protein expression.

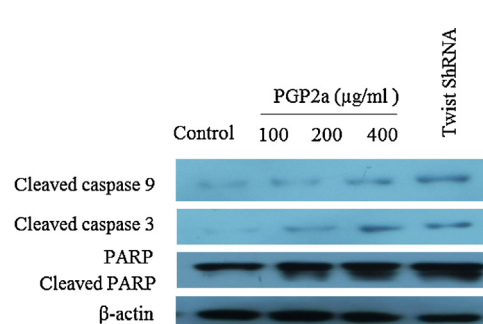


Fig. 5. Effect of PGP2a on caspases 3, 9 and PARP protein expression.

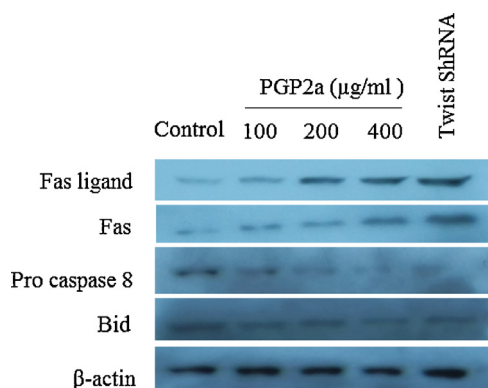


Fig. 6. Effect of PGP2a on Fas, Fas ligand, pro-caspase 8 and Bid protein expression.

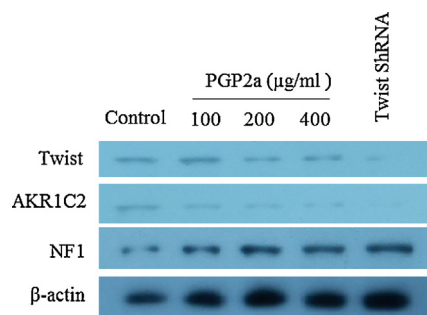


Fig. 7. Effect of PGP2a on Twist, NF-1 and AKR1C2 protein expression.

Fig. 6, we found that the expression of Fas and Fas ligand increased while the levels of both pro caspase 8 and Bid decreased in the PGP2a-treated HGC-27 cells. This response was also observed in Twist-depleted HGC-27 cells. Collectively, these results indicated that death receptor-mediated apoptotic pathways might be also involved in PGP2a induced apoptosis in HGC-27 cells.

3.8. Effect of PGP2a on Twist, NF-1 and AKR1C2 protein expression

Because transcription factor Twist and its two downstream genes (NF-1/AKR1C2) play a pivotal role in control of gastric cancer progression (Feng, Wang, Shi, et al., 2009), we investigated the protein expression of Twist/NF-1/AKR1C2 in HGC-27 cells in response to PGP2a treatment from 100 to 400 µg/mL for 48 h. As shown in Fig. 7, PGP2a induced an increase of NF-1 protein expression, whereas the Twist and AKR1C2 were obviously suppressed by PGP2a. Similarly, Twist knockdown led to a decrease of AKR1C2 and an increase of NF1 in HGC-27 cells. These results suggested that the suppression of Twist expression by PGP2a resulted in the different change of its two downstream proteins of NF1 and AKR1C2, which may at least partly contribute to the mechanism by which PGP2a-induced apoptosis in human gastric cancer HGC-27 cells.

4. Conclusions

Gastric cancer is one of the most common human malignant tumors (Neugut, Hayek, & Howe, 1996). Epidemiological investigations have provided evidence that gastric carcinogenesis is a complex, multistep and multifactorial event (Monari et al., 2009). Due to the unpredictable side effects of the long-term use of commercially available drugs in gastric cancer therapy, the treatment of this painful disease and its prevention has become one of the challenging medical problems of the day (Park, 2002; Wang et al., 2012). It has been reported that natural products from

medicinal herb play a key role in the prevention and termination of development of gastric cancer (Chen et al., 2009; Ishiguro et al., 2007; Miao et al., 2010). In the present work, ginseng polysaccharides were completely fractionated into five fractions by DEAE Sepharose Fast Flow column chromatography. In the light of screening investigation for antitumor polysaccharides, PGP2a was found to have high cytotoxic activity on human gastric cancer HGC-27 cells. Therefore, in our next series of experiments, the antiproliferative effect of PGP2a in gastric cancer HGC-27 cells and its underlying mechanism for the induction of apoptosis were demonstrated.

The percentage of surviving cells is an indicator of the cell proliferative activity. To assess the inhibitory effect of PGP2a on the growth of HGC-27 cells, we first determined the growth rates of AGS cells by the MTT assay. Exponentially growing HGC-27 cells were continuously cultured in the absence or presence of different doses of PGP2a for different time intervals. PGP2a demonstrated a dose-dependent inhibitory effect on cell growth of HGC-27 cells, especially after 48 h co-culture, meaning the activity reached the peak value at 48 h of treatment. This can be interpreted as the result of cell proliferation in prolonged time. When cells grow for a short time, the proliferation fails to arrive at the maximum value. But for a long time, the pre-survived cells had a chance to thrive in a relative nutritional environment, thus causing the longer time, the lower toxicity. More importantly, PGP2a also showed no toxicity on the human normal gastric epithelial AGS cells. Next, the inhibitory effect of PGP2a on HGC-27 cells was confirmed by morphological analysis. The AO/EB fluorescent staining assay showed fragmentation and condensation of chromatin in HGC-27 cells.

More and more investigations indicated that cell cycle arrest and apoptosis or programmed cell death are closely related to the modulation of growth of tumor cell (Trivedi, Roberts, Wolf, & Swanborg, 2005). Flow cytometry analysis showed that PGP2a arrested HGC-27 cells in G2/M phase of the cell cycle and induced a dose-dependent increase in the number of total apoptotic cells. Similar response was also observed in Twist-depleted HGC-27 cells. These results suggested that cell cycle arrest and apoptosis played an important role in the proliferation inhibition of HGC-27 cells by PGP2a.

There are two distinct pathways that initiate apoptosis designated as mitochondrial and death-receptor pathways (Budihardjo, Oliver, Lutter, Luo, & Wang, 1999). We evaluated the expressions of caspases 3, 8, 9, PARP cleavage, bcl-2, bcl-xl, bax, bid, Fas and Fas ligand by Western blot analysis. The hallmarks of mitochondrial-mediated intrinsic apoptotic pathways, such as caspases 3, 9 and PARP, were observed in their activated forms following PGP2a treatment. For death-receptor-mediated extrinsic apoptotic pathways, the expression of Fas and FasL increased significantly while the levels of both pro-caspase-8 and bid protein decreased in the PGP2a-treated HGC-27 cells. Thus we can conclude that the mitochondrial-mediated intrinsic and death-receptor-mediated extrinsic apoptotic pathways are all involved in the PGP2a-induced apoptosis in HGC-27 cells.

As for Twist and its two downstream genes of AKR1C2 and NF1, they could induce tumor progression, cell proliferation and oncogenesis in HGC-27 cells (Feng, Wang, Song, et al., 2009). So we further detected the effect of PGP2a on Twist, NF-1 and AKR1C2 expression at protein levels using Western blotting analysis. The results indicated that exposure to PGP2a induced a low expression of Twist, thus leading to a high level of NF-1 and a decrease of AKR1C2 in HGC-27 cells.

In conclusion, our results imply that PGP2a induced apoptosis in HGC-27 cells is at least partly mediated by modulation of Twist and its downstream gene expression. These data have also opened a new avenue of research to explore a novel

pharmaceutical agent targeting at Twist gene for inhibiting human gastric cancer development.

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