Allergen-removed *Rhus verniciflua* Stokes suppresses invasion and migration of pancreatic cancer cells through downregulation of the JAK/STAT and Src/FAK signaling pathways

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Abstract. Pancreatic cancer is a leading cause of mortality and morbidity worldwide. Due to drug resistance, and the high toxicity and adverse side effects of existing chemotherapeutic drugs, the current treatment of highly aggressive pancreatic cancer is considered inadequate. Allergen-removed *Rhus verniciflua* Stokes (aRVS) has a strong antiproliferative effect in various cancer cells, and due to its low toxicity, it has emerged as an attractive candidate for cancer treatment. However, the potential use of aRVS as a treatment for pancreatic cancer is relatively unexplored. The present study examined the effects of aRVS on the invasion and migration of pancreatic cancer cells, and identified the molecular mechanisms underlying its anticancer effects. aRVS inhibited the Janus kinase/signal transducer and activator of transcription pathway in pancreatic cancer cells, and decreased the protein expression of mucin 4. In addition, it inhibited the activation of focal adhesion kinase and Src signaling, and decreased the expression of matrix metalloproteinase 9, which may reduce the migration and invasion of pancreatic cancer cells. In conclusion, the present study suggested that aRVS may be a potential treatment for aggressive pancreatic cancer.

Introduction

Pancreatic cancer is the third most common cause of cancer-associated mortality in the United States, and is the leading cause of mortality and morbidity worldwide (1). Advances in medical technology and chemotherapy over the past few decades have increased overall survival; however, since long-term survival rates are still low, novel therapies are required (2,3). At present, surgical resection is considered a major treatment for patients with pancreatic cancer. Since pancreatic cancer has no early symptoms, in the majority of patients tumor metastasis is observed before resection of malignant tumors (4,5). Even if surgical resection is performed early, almost all patients experience recurrence following surgery, or eventually reach a pathological state due to metastatic growth (5). Therefore, therapeutic strategies that specifically inhibit or prevent invasion or metastasis may significantly improve the prognosis of pancreatic cancer.

Interferon (IFN)-γ promotes the transcriptional activation of IFN-γ-inducible genes, predominantly through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) intracellular signaling pathway (6). When IFN-γ binds to cell surface receptors, the receptor-associated tyrosine kinases JAK1 and JAK2 are activated. JAKs phosphorylate STAT-1; phosphorylated (p)-STAT1 is dimerized and translocated to the nucleus, where it binds to the IFN-γ-activated site element of IFN-γ responsive genes, thus resulting in gene activation (7). In general, STAT1 is considered a tumor suppressor; however, paradoxical evidence supporting the tumor-promoting function of STAT1 has emerged. Previous studies have demonstrated that extended IFN signaling or constitutive STAT1 signaling promotes tumor growth, as well as resistance to chemotherapy and radiation (8-13). STAT1 enhances cancer cell growth, invasion and exhibits various activities, including inhibition of cell death and induction of therapeutic resistance (14).

Rhus verniciflua Stokes (RVS) is a member of the Anacardiaceae family, which is also known as the lacquer tree, and has been used for the treatment of gastric diseases, including tumors, for centuries in Traditional Korean Medicine (TKM) (15,16). RVS contains phenolic compounds, such as fustin, fisetin, sulfuretin, butein, gallic acid and kaempferol. It has been reported that RVS exhibits antifibrogenic (17), antiproliferative (16,18), antioxidant (19,20) and antitumorigenic (21-23) activities. In clinical use, urushiol should be

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removed from RVS, as it may induce an allergic skin rash in sensitive individuals. Allergen-removed RVS (aRVS) has also been revealed to exert anticancer effects in preclinical studies, and has potential as an anticancer therapeutic agent for the treatment of patients with advanced cancer, including pancreatic cancer (24-26).

Since cancer cell invasion and metastasis involve STAT1 activation and aRVS has been reported to possess antitumorigenic activity, the present study investigated whether aRVS serves a pivotal role in this process. The present results demonstrated that activation and upregulation of STAT1 by IFN-γ initiated the induction of pancreatic cancer cell invasion and metastasis, whereas aRVS suppressed these carcinogenic processes, potentially through inhibition of IFN-γ-induced STAT1 activation.

Materials and methods

Materials. aRVS was obtained from Bflux Pharma Corp (Seoul, South Korea). The voucher specimen was registered and deposited at East-West Medical Research Institute, Kyung Hee University (Seoul, South Korea). The preparative process and quality control of aRVS was conducted according to the standard operational procedure of Bflux Pharma Corp (Korean patent no. 10-2016-0101802) (27). Briefly, RVS stalk, which was 10 years old, was purchased from Kyung Hee Herb Pharm (Wonju, Korea). The dried RVS stalk was chopped into pieces, and extracted twice with a 4-fold volume of purified water at 105-110˚C for 4 h. The extract was filtered using a 1-mm filtration device, and was vacuum concentrated. The concentrate was lyophilized to powder form, resulting in a 3% yield of aRVS extract. Quality control was performed using high-performance liquid chromatography (fisetin, >10.0%; fustin, >8.0%; gallic acid, >8.0%; sulfuretin, >1.5%; urushiol, not detected), a pesticide detection test and a residual microorganism test. aRVS was dissolved in 50% methanol as a 200 mg/ml stock solution and stored at -20˚C. Further dilution was conducted in cell culture medium. Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, fetal bovine serum (FBS), 0.25% trypsin-EDTA and antibiotic-antimycotic (100 U/ml penicillin and streptomycin) were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). MTT, dimethyl sulfoxide (DMSO) and SDS were purchased from Sigma-Aldrich (Merck KgaA, Darmstadt, Germany). Matrigel was obtained from BD Biosciences (Franklin Lakes, NJ, USA), type I collagen was purchased from Costar (Corning Incorporated, Corning, NY, USA), and IFN-γ was purchased from BioLegend, Inc. (San Diego, CA, USA). Antibodies against p-Src (cat. no. 2101), Src (cat. no. 2108), X-linked inhibitor of apoptosis protein (XIAP) (cat. no. 2042), cleaved-caspase-3 (cat. no. 9661), p-JAK1(cat. no. 3331), p-JAK2 (cat. no. 8082), p-STAT1 (Y701, S727) (cat. no. 7649), STAT1 (cat. no. 9172), p-STAT3 (Y705) (cat. no. 9145), p-focal adhesion kinase (FAK) (cat. no. 3284) and FAK (cat. no. 13009), as well as secondary antibodies (anti-rabbit; cat. no. 7074, anti-mouse; cat. no. 7076), were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Matrix metalloproteinase 9 (MMP9) (cat. no. sc-21733), B-cell lymphoma 2 (Bcl2) (cat. no. sc-492), cyclin D1 (cat. no. sc-718), JAK1 (cat. no. sc-277), JAK2 (cat. no. sc-278), STAT3 (cat. no. sc-482) and β-actin (cat. no. sc-47778) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). MUC4 (cat. no. ab60720) antibody was purchased from Abcam (Abcam, Cambridge, MA).

Cell culture. A total of three pancreatic cancer cell lines (PANC-1, HPAC and Bxpc3) were obtained from the American Type Culture Collection (Manassas, VA, USA). The PANC-1 and HPAC immortalized human pancreatic cancer cell lines were cultured in DMEM supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. Bxpc3 pancreatic cancer cells were cultured in RPMI-1640 supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. All cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. After attaining 80% confluence, the cells were subcultured by trypsinization with trypsin-EDTA solution.

Cell viability assay. Cell viability and cell number were determined using an MTT (purity >95%) assay. HPAC ($1x10^4$ cells/well), PANC ($1x10^4$ cells/well) and Bxpc3 ($1x10^4$ cells/well) were respectively seeded into 96-well plates with 100 μl culture medium and were treated with the indicated concentrations (50, 100 and 200 μM) of aRVS for 24 h, and 20 ng/ml IFN-γ for 24 h at 37˚C. Subsequently, 20 μl MTT (5 mg/ml) solution was added to each well, and the cells were incubated for 4 h at 37˚C. Once the medium was carefully removed, 150 μl DMSO was added and agitated to dissolve the formazan crystals. The absorbance at 490 nm was measured using Tecan Sunrise Eliza-Reader (Tecan Group Ltd., Mannedorf, Switzerland). For relative quantification, the value of absorbance in each group was normalized to that of the control group.

Western blotting. Cells were treated with IFN-γ (20 ng/ml) and aRVS (200 μg/ml), or with IFN-γ (20 ng/ml) alone, for 24 or 48 h at 37˚C, and were then washed and collected. Total protein was extracted using radioimmunoprecipitation assay buffer (Cell Signaling Technology Inc.) containing a complete protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany), and proteins were then incubated on ice. After 10 min, cells were centrifuged at 16,000 x g for 10 min at 4˚C, and the protein concentration was measured using the bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Subsequently, 30 μg protein samples were separated by 10% SDS-PAGE, after which, proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST) at room temperature for 1 h. The membrane was washed three times with TBST and incubated with the primary antibodies overnight at 4˚C. The membrane was then washed three times in TBST, and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (dilution 1:2,000; cat. no. 7074) and HRP-conjugated goat anti-mouse secondary antibodies (dilution 1:2,000; cat. no. 7076) (both Cell Signaling Technology, Inc.) for 1 h at room temperature. The immunoreactive proteins were visualized using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (cat. no. 34580) or SuperSignal™ West Dura Extended Duration Substrate (cat. no. 34075) (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols.

Images were captured using an Imagequant™ LAS 4000 (GE Healthcare Japan, Tokyo, Japan).

Dilutions for primary antibodies used in the present study are as follows; MUC4 (1:1,000), p-Src (1:1,000), Src (1:1,000), XIAP (1:1,000), cleaved-caspase-3 (1:1,000), p-JAK1 (1:1,000), p-JAK2 (1:1,000), p-STAT1 (Y701, S727) (1:1,000), STAT1 (1:1,000), p-STAT3 (Y705) (1:1,000), p-FAK (1:1,000), FAK (1:1,000), JAK1 (1:1,000), JAK2 (1:1,000), STAT3 (1:1,000) and β-actin (1:2,000).

Matrigel-invasion assay. The *in vitro* invasion assay was performed using a 24-well Transwell unit (pore size, 8 μm) with polycarbonate membranes (Costar; Corning Incorporated). The upper and lower sides of the membrane were coated with Matrigel (1 mg/ml) and type I collagen (0.5 mg/ml). The lower chamber was filled with 10% FBS-containing medium or serum-free medium containing IFN-γ (20 ng/ml). Cells ($1x10^5$/ml) were placed in the upper chamber of the Transwell unit and were cultured for 18 h at 37˚C with or without aRVS (200 μg/ml). The noninvading cells on the upper surface of membrane were removed from the chamber, and the invading cells on the lower surface of the membrane were stained with Quick-Diff stain kit (BD Biosciences). Briefly, the cells were fixed in REASTAIN Quick-Diff Fix for 5 min, and were then stained with REASTAIN Quick-Diff Red followed by REASTAIN Quick-Diff Blue for 10 min each. The number of invasive cells was counted in five randomly selected fields under a Nikon Ti-U microscope (Nikon Corporation, Tokyo, Japan) at x200 magnification.

Wound healing assay. Once cells reached 80% confluence, they were pretreated with mitomycin C (25 μg/ml) in serum-free medium for 30 min to suppress cell proliferation before a wound was made to the cell monolayer using a 200-μl pipette tip (28,29). In all subsequent experimental steps, medium contained mitomycin C. After washing with serum-free medium, cells were incubated with 10% FBS-containing medium or serum-free medium containing IFN-γ (20 ng/ml) in the presence or absence of aRVS (200 μg/ml) for 24 h at 37˚C. The migration of the cells at the edge of the scratch was analyzed at 0, 18 and 24 h, when microscopic images of the cells were captured. Images were captured using a digital camera system (Nikon Corporation) connected to a light microscope (Olympus America, Inc., Melville, NY, USA) at different time points.

Statistical analysis. The results obtained from each experiment are expressed as the means ± standard deviation from at least three independent experiments. Statistical analysis was performed using one way analysis of variance followed by Tukey's post hoc test for multiple comparisons. GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used to analyze data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

aRVS inhibits cell invasion and migration of pancreatic cancer cells. To evaluate the therapeutic potential of aRVS in pancreatic cancer, its effects on invasion and migration were analyzed using Matrigel invasion and wound healing assays. The preliminary results demonstrated that HPAC cells had the highest metastatic capacity among several pancreatic cancer cell lines (data not shown); therefore, HPAC cells were used for the following experiments. As shown in Fig. 1A, cells treated with aRVS experienced a significant decrease in the number of invading cells compared with those treated with 10% FBS. The wound healing assay also indicated that 10% FBS-treated cells migrated across the wound much faster than aRVS-treated cells (Fig. 1B). These results indicated that

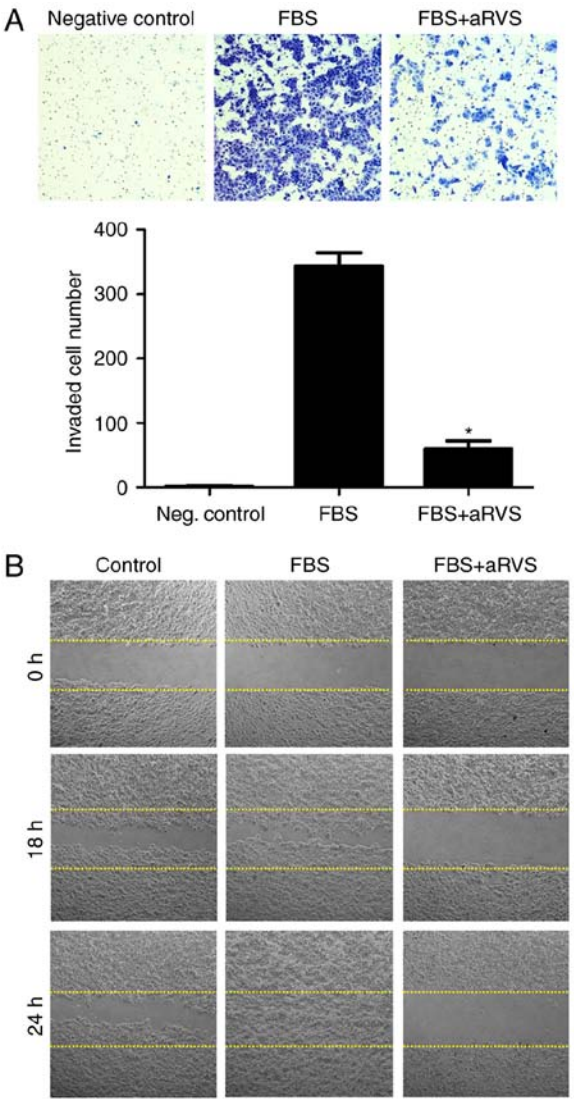

Figure 1. aRVS inhibits cell migration and invasion of pancreatic cancer cells. (A) Matrigel-invasion assay determined the inhibitory effects of aRVS on HPAC pancreatic cancer cell invasion. HPAC cells were treated with 10% FBS and aRVS, or 10% FBS only, and an equal number of cells was seeded into the upper chamber of a Matrigel-coated Transwell system. After 18 h at 37˚C, non-invading cells on the upper part of the membrane were removed with a cotton swab, and the invasive cells were fixed and stained. The number of cells was quantified in five random fields. Quantitative results were obtained from x200 magnification images (*P<0.01 vs. the FBS-treated group). (B) Wound healing assay indicated the effects of aRVS on the mobility of HPAC cells. Confluent cells were scratched with a 200-μl sterile pipette tip at the center of the well, and cells were then treated with 10% FBS and aRVS, or with 10% FBS alone for 24 h at 37˚C. Images of the wounded monolayer were captured at the indicated times via a light microscope under x100 magnification. aRVS, allergen-removed *Rhus verniciflua* Stokes; FBS, fetal bovine serum.

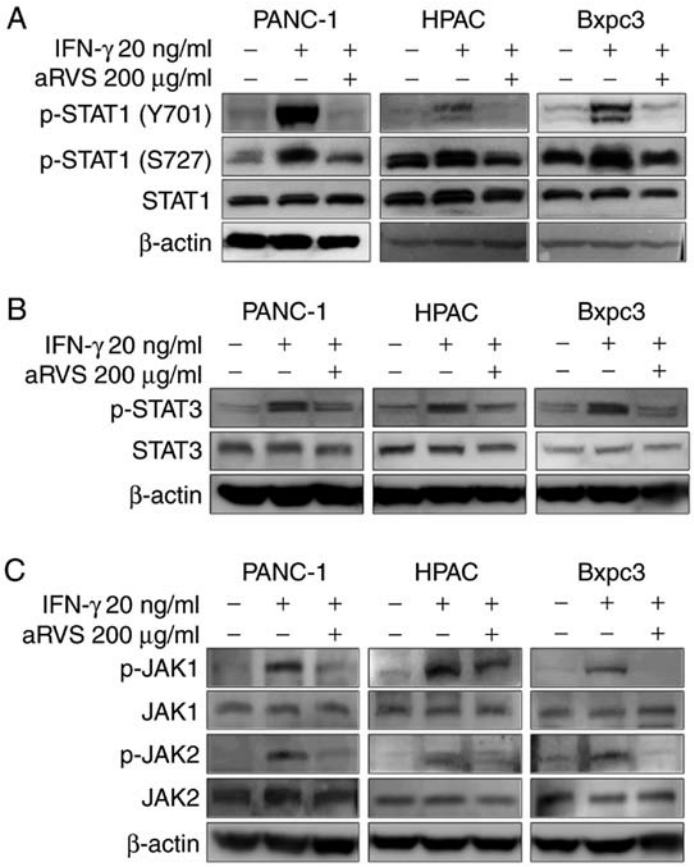

Figure 2. aRVS downregulates the IFN-γ-induced JAK/STAT pathway in pancreatic cancer cells. (A-C) Cells were treated with IFN-γ and aRVS, or with IFN-γ alone for 24 h and whole cell extracts were collected. Protein expression was determined by western blotting with antibodies against phosphorylated and total STAT and JAK proteins. β-actin was used as a loading control. Representative data of three independent experiments are shown. aRVS, allergen-removed *Rhus verniciflua* Stokes; IFN-γ, interferon-γ; JAK, Janus kinase; p-, phosphorylated; STAT, signal transducer and activator of transcription.

aRVS significantly inhibited the invasion and migration of HPAC pancreatic cancer cells.

aRVS downregulates the IFN-γ-induced STAT1 signaling pathway. STAT1 and STAT3 transcription factors serve an important role in tumorigenesis. Despite the controversial nature of the effects of STAT1 on tumorigenesis, STAT3 strongly induces cancer invasion or migration (14,30,31). Therefore, the present study examined whether aRVS regulated the activity of STAT1 or STAT3, thus leading to tumor invasion and migration. The results demonstrated that aRVS inhibited the IFN-γ-induced activation of STAT1 and STAT3; however, STAT1 was more strongly inhibited by aRVS in IFN-γ-stimulated PANC-1, HPAC and Bxpc3 cells (Fig. 2A and B). In the process of STAT1 activation, the phosphorylation of Ser727 is required for the dimerization of STAT1, and the phosphorylation of Tyr701 is essential for translocation of STAT1 into the nucleus (32,33). Upon examination, the present study demonstrated that aRVS inhibited the phosphorylation of both serine and tyrosine residues, thus suggesting that aRVS may block the phosphorylation of both residues. However, the suppression of Tyr701 phosphorylation in STAT1 by aRVS was more marked than that of Ser727. Based on these results, the present study aimed to determine whether aRVS inhibited the upstream kinases of the STAT signaling pathway. As shown in Fig. 2C, aRVS also inhibited the activity of JAK1 and JAK2.

aRVS inhibits IFN-γ-induced invasion, migration and proliferation. The present study also investigated whether the activation of STAT1 by IFN-γ induced invasion, migration and proliferation of pancreatic cancer cells, and examined whether this was inhibited by aRVS. The results demonstrated that IFN-γ induced the invasion, migration and proliferation of pancreatic cancer cells, and that aRVS treatment significantly inhibited these effects (Fig. 3). The wound healing assay indicated that IFN-γ-treated cells migrated much faster than cells treated with aRVS (Fig. 3B). In the invasion assay, treatment with aRVS resulted in a significantly reduced number of invasive cells compared with in the IFN-γ-treated group (Fig. 3A). In addition, aRVS significantly inhibited IFN-γ-induced proliferation of pancreatic cancer cells in a concentration-dependent manner (Fig. 3C). The present study also confirmed that the inhibitory effects of aRVS were not due to toxicity (data not shown).

Our preliminary experiments revealed that HPAC cell mobility was the most abundant and aRVS exhibited potent inhibitory effects on all three pancreatic cell lines. Subsequently, the expression levels of MMP9, which targets various extracellular proteins during invasion and metastasis, were analyzed, in order to confirm the ability of aRVS to inhibit the invasion and metastasis of pancreatic cancer cells. The results indicated that the expression levels of MMP9 were lower in cells treated with aRVS compared with in those treated with IFN-γ (Fig. 4). Based on these results, it was

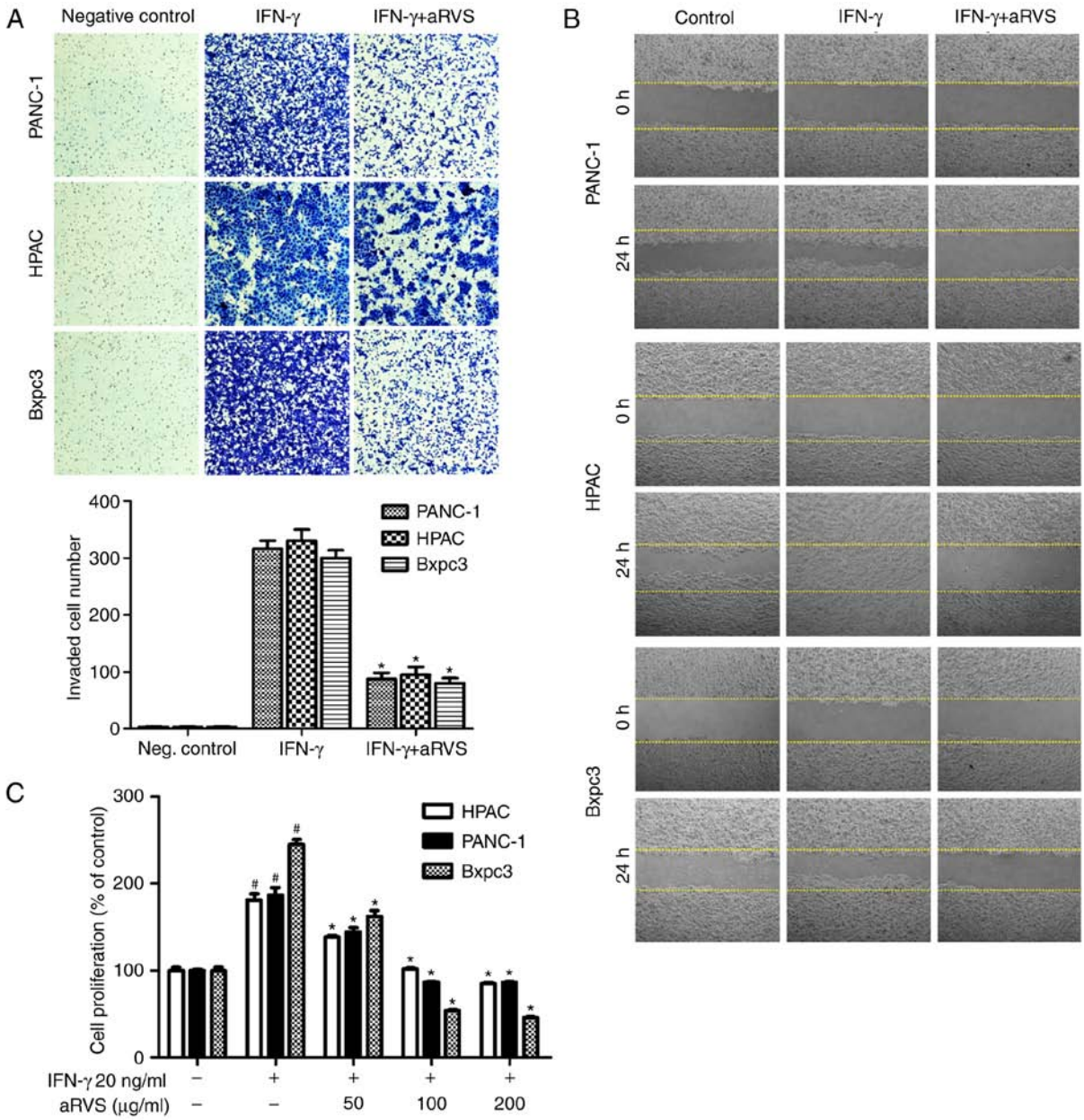

Figure 3. aRVS suppresses IFN-γ-induced invasion and migration of various pancreatic cancer cells. (A) Matrigel-invasion assay determined the inhibitory effects of aRVS on PANC-1, HPAC and Bxpc3 pancreatic cancer cell invasion. Cells were treated with IFN-γ and aRVS, or with IFN-γ alone, and equal numbers of the cells were seeded into the upper chamber of a Matrigel-coated Transwell system. After 18 h at 37˚C, non-invading cells on the upper part of the membrane were removed with a cotton swab, and the invasive cells were fixed and stained. The number of cells was quantified in five random fields. Quantitative results were obtained from x200 magnification images. *P<0.01 vs. the IFN-γ- treated group. (B) Wound healing assay determined the effects of aRVS on the migratory ability of PANC-1, HPAC and Bxpc3 cells. Confluent cells were scratched with a 200-μl sterile pipette tip at the center of the well, and cells were treated with IFN-γ and aRVS, or with IFN-γ alone for 24 h at 37˚C. Images of the wounded monolayer were captured at the indicated time points under a light microscope with x100 magnification. (C) Serum-starved cells ($1x10^4$ cells) were stimulated with IFN-γ in the presence or absence of aRVS, and the number of viable cells was measured using the MTT assay. #P<0.05 vs. the Control group; *P<0.05 vs. the IFN-γ-treated group. Data are presented as the means ± standard error of the mean from three independent experiments. aRVS, allergen-removed *Rhus verniciflua* Stokes; IFN-γ, interferon-γ.

suggested that aRVS may inhibit the invasion and metastasis of pancreatic cancer cells via the inhibition of STAT1.

aRVS downregulates mucin 4 (MUC4) expression in pancreatic cancer cells. It has previously been indicated the involvement of MUC4 in pancreatic cancer cell motility and invasion; furthermore, it promotes resistance to apoptosis when cells are treated with various chemotherapeutic agents (34). In addition, the activated transcription factors STAT1 and STAT3 have been reported to serve as potential regulators of MUC4 expression in pancreatic cancer cells (35). Based on these reports, the present study determined whether aRVS could affect MUC4 expression in pancreatic cancer cells. PANC-1, HPAC and Bxpc3 cells were untreated for 48 h, or were treated with IFN-γ with or without aRVS for 24 and 48 h. The cells were then harvested and MUC4 expression was evaluated using western blotting. aRVS reduced MUC4 expression in the three pancreatic cancer cell lines; MUC4 was almost eliminated following treatment with aRVS for 48 h (Fig. 4).

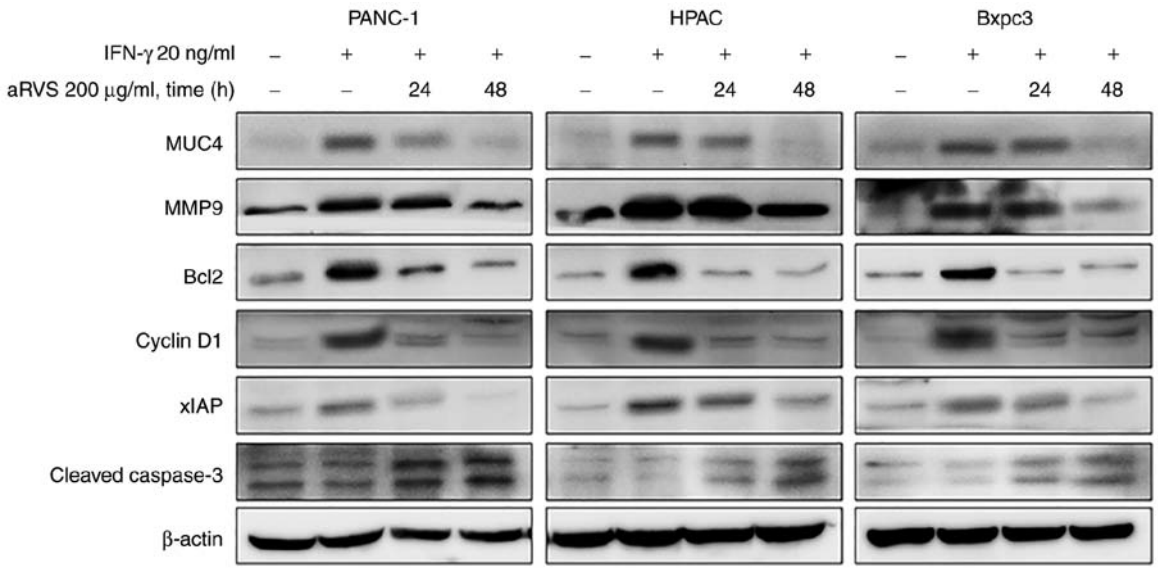

Figure 4. aRVS regulates the expression of MUC4, and anti-apoptotic and cell cycle regulatory proteins. Cells were treated with IFN-γ and aRVS, or with IFN-γ alone for the indicated time points, and whole cell extracts were collected. Protein expression was determined by western blotting with antibodies against MUC4, MMP9, Bcl2, cyclin D1, xIAP and cleaved caspase-3 proteins. β-actin was used as a loading control. Representative data of three independent experiments are shown. aRVS, allergen-removed *Rhus verniciflua* Stokes; Bcl2, B-cell lymphoma 2; IFN-γ, interferon-γ; MMP9, matrix metalloproteinase 9; MUC4, mucin 4; xIAP, X-linked inhibitor of apoptosis protein.

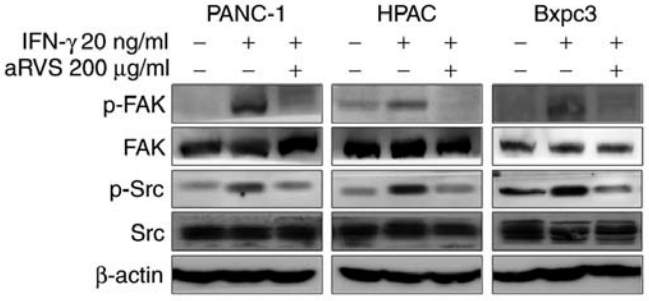

Figure 5. aRVS downregulates FAK/Src signaling in pancreatic cancer cells. Cells were treated with IFN-γ and aRVS, or with IFN-γ alone for 24 h and whole cell extracts were prepared. Protein expression was analyzed by western blotting with antibodies against phosphorylated and total FAK and Src proteins. The same membranes were stripped and reincubated with anti-β-actin to confirm equal protein loading. Representative data of three independent experiments are shown. aRVS, allergen-removed *Rhus verniciflua* Stokes; FAK, focal adhesion kinase; IFN-γ, interferon-γ; p-, phosphorylated.

aRVS downregulates cell cycle regulatory and anti-apoptotic proteins. The present study also examined the effects of aRVS on the expression levels of various proteins associated with cell cycle regulation and apoptosis. Treatment with aRVS reduced the expression of the cell cycle regulatory protein cyclin D1. In addition, the expression levels of the anti-apoptotic molecules Bcl2 and XIAP were decreased by aRVS, whereas the expression of the cleaved form of the proapoptotic molecule caspase-3 was increased (Fig. 4). These results suggested that aRVS may modulate the cell cycle and the intrinsic mitochondrial apoptotic pathway in pancreatic cancer cells.

aRVS downregulates FAK and Src signaling. The FAK protein has an important role in cellular motility and invasion; recently, studies have been conducted regarding the association between FAK and STAT1 (36,37). In addition, the phosphorylation and activation of FAK by Src is required for actin stress fiber formation, and for focal adhesion assembly during cell adhesion and cell spreading. Therefore, the present study aimed to determine whether aRVS was involved in the activities of FAK and Src. The results demonstrated that FAK and Src were activated by IFN-γ, whereas aRVS reduced the phosphorylation of FAK and Src in PANC-1, HPAC and Bxpc3 cells (Fig. 5).

Discussion

RVS and aRVS have been reported to induce apoptosis, inhibit angiogenesis, and possess antioxidant and antiproliferative activities (20,24,26,38,39). aRVS and RVS consist of multiple constituents, including phenolic acids (gallic acid, protocatechuic acid, etc.), flavonoids (fisetin, sulfuretin, fustin, butein, quercetin, etc.), and other constituents (chlorogenic acid, kaempferol-3-O-glucoside, p-coumaric acid, etc.). Kim *et al* previously described the chemical structures of the aforementioned constituents (26). In TKM, aRVS has a long history of use due to its various efficacies and low toxicity; however, there is still uncertainty about the specific mechanism of aRVS treatment for the treatment of patients with cancer. Therefore, the present study evaluated the anticancer effect of aRVS using various pancreatic cancer cell lines. The present study indicated that aRVS treatment affected the regulation of MUC4 and FAK expression via the inhibition of JAK/STAT1 signaling, thus potentially reducing invasion and metastasis. To the best of our knowledge, the present study is the first to demonstrate that aRVS may modulate MUC4 and identified MUC4 as a potential therapeutic target for pancreatic cancer. Therefore, mucin expression may have an important role in carcinogenesis progression of pancreatic cancer. This finding may be valuable for a large number of patients with pancreatic

cancer, in whom significant overexpression of MUC4 has been detected in pancreatic cancer compared with normal pancreatic expression (34).

One of the notable discoveries of the present study is the modulation of MUC4. MUC4 is a member of the membrane-binding mucins, which is known to be overexpressed in pancreatic cancer cells. Conversely, it is not expressed in normal pancreatic tissue, whereas its expression is steadily increased with the stage of disease progression and is associated with a poor prognosis (40,41). Other studies have also reported the oncogenic role of MUC4 and revealed that MUC4 induces the survival, invasion and metastasis of pancreatic cancer (42,43). MUC4 also induces epithelial-to-mesenchymal transition through the stabilization of N-cadherin expression in pancreatic cancer cells (44). Not only MUC4, but also MUC1 and MUC16, are overexpressed in pancreatic cancer cells and contribute to its pathogenesis (45,46). Considering the importance of MUC4 in the pathogenesis of pancreatic cancer, the present study identified a downregulation mechanism of MUC4 induced by aRVS.

Several cytokines, including interleukin (IL)-4, IL-6, tumor necrosis factor-α and IFN-γ, have been reported to be involved in overexpression of MUC4 via the JAK/STAT pathway, particularly STAT1 and STAT3 (47). Previous studies regarding the MUC4 promoter have identified the binding sites for several transcription factors, including STAT1 and STAT3 (34,35,48). These findings suggested that the STAT transcription factors may have an important role in the transcriptional regulation of MUC4. In the present study, STAT1 and STAT3 phosphorylation, and MUC4 expression were reduced in pancreatic cells treated with aRVS; furthermore, the phosphorylation of the upstream kinases JAK1 and JAK2 were also reduced. These results suggested that the inhibition of MUC4 by aRVS may have an effect on STAT expression.

The induction of apoptosis and the inhibition of cell proliferation are important mechanisms underlying the anticancer action of numerous drugs from natural sources (49). The present study revealed that aRVS treatment inhibited cell proliferation and induced apoptosis, via the activation of caspase-3 and the suppression of Bcl-2, XIAP and cyclin D1. These results indicated that aRVS may be involved in the intrinsic apoptosis pathway. The downregulation of MUC4 has also been revealed to induce the intrinsic pathway of apoptosis (50), which may be a possible mechanism by which aRVS induces apoptosis in pancreatic cancer cells.

Cell motility is an important process in tumor invasion and is an attractive therapeutic target for advanced pancreatic cancer. Previous studies have reported that MUC4 modulates the mobility, morphology and actin-cytoskeleton of cancer cells (42,51). In the present study, aRVS inhibited the mobility and invasion of pancreatic cancer cells, and the results suggested that the inhibitory effects of aRVS may be caused by downregulation of MUC4. FAK is also overexpressed in invasive tumors, and induces the invasion and metastasis of cancer cells, in addition to re-organization of the cytoskeleton and MMPs (52). The present study revealed that aRVS inhibited the phosphorylation of FAK and Src, without altering total FAK and Src levels. Therefore, it may be suggested that aRVS reduces cell mobility and invasion by regulating FAK and Src signaling through STAT1 and MUC4.

In conclusion, the present study is the first, to the best of our knowledge, to report that aRVS downregulated MUC4. In addition, this study provides further evidence regarding the molecular mechanism underlying the effects of aRVS on pancreatic cancer. Considering the overexpression of MUC4, and its association with chemotherapeutic resistance, in pancreatic cancer (50,53), MUC4-targeted chemotherapy using aRVS may be a potential therapeutic strategy. Overall, aRVS may serve an important role in the downregulation of MUC4 and offers a potential for the development of novel therapies for pancreatic cancer. Further studies are required to assess the therapeutic value of aRVS in preclinical models.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

SWY and BP designed the research. YK performed the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2015. CA Cancer J Clin 65: 5-29, 2015.
2. Conroy T, Desseigne F, Ychou M, Bouché O, Guimbaud R, Bécouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardière C, *et al*: FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 364: 1817-1825, 2011.
3. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, Seay T, Tjulandin SA, Ma WW, Saleh MN, *et al*: Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 369: 1691-1703, 2013.
4. Warshaw AL and Fernández-del Castillo C: Pancreatic carcinoma. N Engl J Med 326: 455-465, 1992.

5. Li D, Xie K, Wolff R and Abbruzzese JL: Pancreatic cancer. Lancet 363: 1049-1057, 2004.
6. Stark GR: How cells respond to interferons revisited: From early history to current complexity. Cytokine Growth Factor Rev 18: 419-423, 2007.
7. Darnell JE Jr, Kerr IM and Stark GR: Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264: 1415-1421, 1994.
8. Khodarev NN, Beckett M, Labay E, Darga T, Roizman B and Weichselbaum RR: STAT1 is overexpressed in tumors selected for radioresistance and confers protection from radiation in transduced sensitive cells. Proc Natl Acad Sci USA 101: 1714-1719, 2004.
9. Khodarev NN, Minn AJ, Efimova EV, Darga TE, Labay E, Beckett M, Mauceri HJ, Roizman B and Weichselbaum RR: Signal transducer and activator of transcription 1 regulates both cytotoxic and prosurvival functions in tumor cells. Cancer Res 67: 9214-9220, 2007.
10. Khodarev NN, Roach P, Pitroda SP, Golden DW, Bhayani M, Shao MY, Darga TE, Beveridge MG, Sood RF, Sutton HG, *et al*: STAT1 pathway mediates amplification of metastatic potential and resistance to therapy. PLoS One 4: e5821, 2009.
11. Weichselbaum RR, Ishwaran H, Yoon T, Nuyten DS, Baker SW, Khodarev N, Su AW, Shaikh AY, Roach P, Kreike B, *et al*: An interferon-related gene signature for DNA damage resistance is a predictive marker for chemotherapy and radiation for breast cancer. Proc Natl Acad Sci USA 105: 18490-18495, 2008.
12. Pitroda SP, Wakim BT, Sood RF, Beveridge MG, Beckett MA, MacDermed DM, Weichselbaum RR and Khodarev NN: STAT1-dependent expression of energy metabolic pathways links tumour growth and radioresistance to the Warburg effect. BMC Med 7: 68, 2009.
13. Kharma B, Baba T, Matsumura N, Kang HS, Hamanishi J, Murakami R, McConechy MM, Leung S, Yamaguchi K, Hosoe Y, *et al*: STAT1 drives tumor progression in serous papillary endometrial cancer. Cancer Res 74: 6519-6530, 2014.
14. Meissl K, Macho-Maschler S, Müller M and Strobl B: The good and the bad faces of STAT1 in solid tumours. Cytokine 89: 12-20, 2017.
15. Son YO, Lee KY, Lee JC, Jang HS, Kim JG, Jeon YM and Jang YS: Selective antiproliferative and apoptotic effects of flavonoids purified from *Rhus verniciflua* Stokes on normal versus transformed hepatic cell lines. Toxicol Lett 155: 115-125, 2005.
16. Jang HS, Kook SH, Son YO, Kim JG, Jeon YM, Jang YS, Choi KC, Kim J, Han SK, Lee KY, *et al*: Flavonoids purified from *Rhus verniciflua* Stokes actively inhibit cell growth and induce apoptosis in human osteosarcoma cells. Biochim Biophys Acta 1726: 309-316, 2005.
17. Lee SH, Nan JX, Zhao YZ, Woo SW, Park EJ, Kang TH, Seo GS, Kim YC and Sohn DH: The chalcone butein from *Rhus verniciflua* shows antifibrogenic activity. Planta Med 69: 990-994, 2003.
18. Samoszuk M, Tan J and Chorn G: The chalcone butein from *Rhus verniciflua* Stokes inhibits clonogenic growth of human breast cancer cells co-cultured with fibroblasts. BMC Complement Altern Med 5: 5, 2005.
19. Lee JC, Lim KT and Jang YS: Identification of *Rhus verniciflua* Stokes compounds that exhibit free radical scavenging and anti-apoptotic properties. Biochim Biophys Acta 1570: 181-191, 2002.
20. Lim KT, Hu C and Kitts DD: Antioxidant activity of a *Rhus verniciflua* Stokes ethanol extract. Food Chem Toxicol 39: 229-237, 2001.
21. Kitts DD and Lim KT: Antitumorigenic and cytotoxic properties of an ethanol extract derived from Rhus verniciflua Stokes (RVS). J Toxicol Environ Health A 64: 357-371, 2001.
22. Lee JC, Kim J and Jang YS: Ethanol-eluted extract of *Rhus verniciflua* stokes inhibits cell growth and induces apoptosis in human lymphoma cells. J Biochem Mol Biol 36: 337-343, 2003.
23. Lim KT, Lee SJ, Heo KS and Lim K: Effects of glycoprotein isolated from *Rhus verniciflua* stokes on TPA-induced apoptosis and production of cytokines in cultured mouse primary splenocytes. Toxicol Lett 145: 261-271, 2003.
24. Choi W, Jung H, Kim K, Lee S, Yoon S, Park J, Kim S, Cheon S, Eo W and Lee S: *Rhus verniciflua* stokes against advanced cancer: A perspective from the Korean Integrative Cancer Center. J Biomed Biotechnol 2012: 874276, 2012.
25. Lee S, Kim K, Jung H, Lee S, Cheon S, Kim S, Eo W and Choi W: Efficacy and safety of standardized allergen-removed *Rhus verniciflua* Stokes extract in patients with advanced or metastatic pancreatic cancer: A Korean single-center experience. Oncology 81: 312-318, 2011.
26. Kim JH, Shin YC and Ko SG: Integrating traditional medicine into modern inflammatory diseases care: Multitargeting by *Rhus verniciflua* Stokes. Mediators Inflamm 2014: 154561, 2014.
27. Ryu JC: Toxicity free lacquer solution and device for producing the same. Korean Patent 10-2016-0101802. Filed August 10, 2016; issued August 10, 2016.
28. Nyegaard S, Christensen B and Rasmussen JT: An optimized method for accurate quantification of cell migration using human small intestine cells. Metab Eng Commun 3: 76-83, 2016.
29. Wang Z, Wang Y, Farhangfar F, Zimmer M and Zhang Y: Enhanced keratinocyte proliferation and migration in co-culture with fibroblasts. PLoS One 7: e40951, 2012.
30. Teng Y, Ross JL and Cowell JK: The involvement of JAK-STAT3 in cell motility, invasion, and metastasis. JAKSTAT 3: e28086, 2014.
31. Bu LL, Deng WW, Huang CF, Liu B, Zhang WF and Sun ZJ: Inhibition of STAT3 reduces proliferation and invasion in salivary gland adenoid cystic carcinoma. Am J Cancer Res 5: 1751-1761, 2015.
32. Barnholt KE, Kota RS, Aung HH and Rutledge JC: Adenosine blocks IFN-gamma-induced phosphorylation of STAT1 on serine 727 to reduce macrophage activation. J Immunol 183: 6767-6777, 2009.
33. Kovarik P, Stoiber D, Eyers PA, Menghini R, Neininger A, Gaestel M, Cohen P and Decker T: Stress-induced phosphorylation of STAT1 at Ser727 requires p38 mitogen-activated protein kinase whereas IFN-gamma uses a different signaling pathway. Proc Natl Acad Sci USA 96: 13956-13961, 1999.
34. Andrianifahanana M, Singh AP, Nemos C, Ponnusamy MP, Moniaux N, Mehta PP, Varshney GC and Batra SK: IFN-gamma-induced expression of MUC4 in pancreatic cancer cells is mediated by STAT-1 upregulation: A novel mechanism for IFN-gamma response. Oncogene 26: 7251-7261, 2007.
35. Perrais M, Pigny P, Ducourouble MP, Petitprez D, Porchet N, Aubert JP and Van Seuningen I: Characterization of human mucin gene MUC4 promoter: Importance of growth factors and proinflammatory cytokines for its regulation in pancreatic cancer cells. J Biol Chem 276: 30923-30933, 2001.
36. Xie B, Zhao J, Kitagawa M, Durbin J, Madri JA, Guan JL and Fu XY: Focal adhesion kinase activates Stat1 in integrin-mediated cell migration and adhesion. J Biol Chem 276: 19512-19523, 2001.
37. Zhang L and Zou W: Inhibition of integrin β1 decreases the malignancy of ovarian cancer cells and potentiates anticancer therapy via the FAK/STAT1 signaling pathway. Mol Med Rep 12: 7869-7876, 2015.
38. Choi HS, Kim MK, Choi YK, Shin YC, Cho SG and Ko SG: *Rhus verniciflua* Stokes (RVS) and butein induce apoptosis of paclitaxel-resistant SKOV-3/PAX ovarian cancer cells through inhibition of AKT phosphorylation. BMC Complement Altern Med 16: 122, 2016.
39. Choi HS, Seo HS, Kim SR, Choi YK, Jang BH, Shin YC and Ko SG: Anti-inflammatory and anti-proliferative effects of *Rhus verniciflua* Stokes in RAW264.7 cells. Mol Med Rep 9: 311-315, 2014.
40. Andrianifahanana M, Moniaux N, Schmied BM, Ringel J, Friess H, Hollingsworth MA, Büchler MW, Aubert JP and Batra SK: Mucin (*MUC*) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: A potential role of MUC4 as a tumor marker of diagnostic significance. Clin Cancer Res 7: 4033-4040, 2001.
41. Chauhan SC, Singh AP, Ruiz F, Johansson SL, Jain M, Smith LM, Moniaux N and Batra SK: Aberrant expression of MUC4 in ovarian carcinoma: Diagnostic significance alone and in combination with MUC1 and MUC16 (CA125). Mod Pathol 19: 1386-1394, 2006.
42. Chaturvedi P, Singh AP, Moniaux N, Senapati S, Chakraborty S, Meza JL and Batra SK: MUC4 mucin potentiates pancreatic tumor cell proliferation, survival, and invasive properties and interferes with its interaction to extracellular matrix proteins. Mol Cancer Res 5: 309-320, 2007.
43. Bafna S, Singh AP, Moniaux N, Eudy JD, Meza JL and Batra SK: MUC4, a multifunctional transmembrane glycoprotein, induces oncogenic transformation of NIH3T3 mouse fibroblast cells. Cancer Res 68: 9231-9238, 2008.

44. Rachagani S, Macha MA, Ponnusamy MP, Haridas D, Kaur S, Jain M and Batra SK: MUC4 potentiates invasion and metastasis of pancreatic cancer cells through stabilization of fibroblast growth factor receptor 1. Carcinogenesis 33: 1953-1964, 2012.
45. Hollingsworth MA and Swanson BJ: Mucins in cancer: Protection and control of the cell surface. Nat Rev Cancer 4: 45-60, 2004.
46. Haridas D, Chakraborty S, Ponnusamy MP, Lakshmanan I, Rachagani S, Cruz E, Kumar S, Das S, Lele SM, Anderson JM, *et al*: Pathobiological implications of MUC16 expression in pancreatic cancer. PLoS One 6: e26839, 2011.
47. Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G and Schaper F: Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J 374: 1-20, 2003.
48. Kossow C, Jose D, Jaster R, Wolkenhauer O and Rateitschak K: Mathematical modelling unravels regulatory mechanisms of interferon-γ-induced STAT1 serine-phosphorylation and MUC4 expression in pancreatic cancer cells. IET Syst Biol 6: 73-85, 2012.
49. Shishodia S and Aggarwal BB: Guggulsterone inhibits NF-kappaB and IkappaBalpha kinase activation, suppresses expression of anti-apoptotic gene products, and enhances apoptosis. J Biol Chem 279: 47148-47158, 2004.
50. Bafna S, Kaur S, Momi N and Batra SK: Pancreatic cancer cells resistance to gemcitabine: The role of MUC4 mucin. Br J Cancer 101: 1155-1161, 2009.
51. Ponnusamy MP, Singh AP, Jain M, Chakraborty S, Moniaux N and Batra SK: MUC4 activates HER2 signalling and enhances the motility of human ovarian cancer cells. Br J Cancer 99: 520-526, 2008.
52. Chang YM, Kung HJ and Evans CP: Nonreceptor tyrosine kinases in prostate cancer. Neoplasia 9: 90-100, 2007.
53. Skrypek N, Duchêne B, Hebbar M, Leteurtre E, van Seuningen I and Jonckheere N: The MUC4 mucin mediates gemcitabine resistance of human pancreatic cancer cells via the concentrative nucleoside transporter family. Oncogene 32: 1714-1723, 2013.