

# PSK induces apoptosis through the inhibition of activated STAT3 in human esophageal carcinoma cells

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**Abstract.** PSK, a protein-bound polysaccharide, is widely used in Japan as an immunopotentiating biological response modifier for cancer patients. PSK exerts antitumor activities through stimulation of the host's immune response; however, few studies have addressed the direct actions of PSK on tumor cells. Recently, it has been found that STAT3 is aberrantly activated in various types of malignancies, and plays a crucial role in tumor cell proliferation and survival. In the present study, STAT3 was constitutively activated in KYSE170 and TE13 esophageal carcinoma cells, and PSK inhibited proliferation and induced apoptosis in these cells in a dose-dependent manner. Based on these findings, the relationship between STAT3 and apoptosis in these cells was investigated. Results showed that PSK inhibited the expression of activated STAT3 and stimulated the expression of pro-apoptotic Bax in a dose-dependent manner, without affecting the expression of anti-apoptotic Bcl-xL and Mcl-1. These results indicate that PSK may induce apoptosis in esophageal carcinoma cells by inhibiting the expression of activated STAT3.

## Introduction

Esophageal cancer is one of the most malignant tumors with a highly aggressive behavior and tends to show systemic spread from an early stage, resulting in a high incidence of recurrence and poor prognosis, even if a complete resection is performed (1). Recently, chemotherapy or chemoradiotherapy has been actively performed as a preoperative or induction therapy in multimodal treatment for advanced tumors, however, the treatment outcome has not been satisfactory (2). On the other hand, tumor progression worsens the host's nutritional state and immune response, which may reduce the therapeutic intensity of conventional treat-

ments (3). In addition, treatment-associated invasiveness may decrease the host's immune response and therapeutic efficacy. In such a situation, the expectation of new treatment modalities, such as molecular target therapy, has been increasing to improve the treatment outcome of esophageal cancer (4).

Polysaccharide-K (PSK), a protein-bound polysaccharide derived from the *Coriolaceae* mushroom, has been used as a non-specific immunostimulant for treating cancer patients in Japan for more than 30 years (5). The antitumor activity for PSK has been documented in experimental animal models and its beneficial therapeutic effects have been shown in clinical studies of several types of tumor (6). PSK has been shown to exert antitumor effects mainly through its action on the host's immune response. On the other hand, PSK has been reported to act on tumor cells themselves, thereby inducing antitumor effects (6). However, the direct action of PSK on tumor cells has been not fully understood in comparison with its action on the host's immune response.

Signal transducer and activator of transcription 3 (STAT3) has a role as a cytoplasmic mediator of cytokine signaling and nuclear transcription factor (7). The activation of STAT3 is tightly regulated under physiologic conditions in normal cells (8). Recently, it was shown that STAT3 is aberrantly activated in various types of malignancies such as leukemia, lymphoma, multiple myeloma, head and neck, breast, and prostate cancers, and plays a crucial role in tumor cell proliferation and survival, and tumor angiogenesis and invasion (9-18). In addition, recent study by Zhang *et al* demonstrated that PSK augments docetaxel-induced apoptosis by inhibiting NF- $\kappa$ B in human pancreatic cancer cells (19). STAT3 regulates a variety of cellular behaviors in close association with NF- $\kappa$ B (20). These findings strongly suggest that PSK may exert antitumor activities by acting not only on NF- $\kappa$ B, but also STAT3.

We performed an *in vitro* study to investigate the direct antitumor effects of PSK and its molecular mechanisms in esophageal carcinoma cell lines; the effect of PSK on cell proliferation and apoptosis focusing on its possible influence on STAT3 status.

## Materials and methods

**Cell lines and cell culture.** The human esophageal squamous cell carcinoma cell lines, KYSE170 and TE13, were obtained from

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the Cell Resource Center for the Biomedical Research Institute of Development, Aging, and Cancer (Tohoku University, Sendai, Japan). Cells were maintained in a culture medium consisting of RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin (Sigma, Welwyn Garden City, UK), and 10% heat-inactivated fetal bovine serum (FBS; Cell Culture Bioscience, Nichirei Biosciences, Tokyo, Japan) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Reagents.** A PSK 5% solution was generously provided by Kureha Chemical Industry (Tokyo, Japan). It was diluted to concentrations ranging from 10 to 500  $\mu$ g/ml by culture medium. The primary antibodies were a rabbit monoclonal anti-phospho-STAT3 (Tyr705) antibody (Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-STAT3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit monoclonal anti-Bcl-xL antibody (Cell Signaling Technology), rabbit monoclonal anti-Mcl-1 antibody (Cell Signaling Technology), mouse monoclonal anti-Bax antibody (Santa Cruz Biotechnology), and rabbit polyclonal anti-GAPDH antibody (Santa Cruz Biotechnology).

**Cell proliferation assay.** Cell proliferation ability was evaluated by counting the number of cells in a 3-day cell culture. Cells (1x10<sup>5</sup> cells) were seeded in 6-well plates in 3-ml culture medium and incubated in the presence of PSK (0, 10, 100, 500  $\mu$ g/ml) for 24, 48, and 72 h. Cells were then collected and the number of cells was counted.

**Cell viability assay.** To examine the sensitivities of human esophageal carcinoma cell lines to PSK, a WST-8 colorimetric assay was performed. Cells seeded in 96-well plates (1x10<sup>3</sup> cells/well) were incubated in the presence of PSK (0, 10, 100, 500  $\mu$ g/ml) for 72 h. After incubation, 10  $\mu$ l of SP solution (Nacalai Tesque) was added to each well and the cells were incubated for 1 h. Cell viability was determined by colorimetric comparison in which optical density values were read by an ELISA reader (GENios, TECAN Group Ltd., Austria) at a wavelength of 450 nm.

**Flow cytometry for cell cycle analysis.** Cells (1x10<sup>5</sup> cells) were seeded in 6-well plates in 3-ml culture medium and pre-incubated for 24 h. PSK (0, 100  $\mu$ g/ml) was added to each well and incubated for 72 h. Cells and culture medium were retrieved from each well to 15-ml conical tubes and were centrifuged for 5 min at 1,500 rpm. They were mixed with 0.2% Triton X-100 (Nacalai Tesque) and 1 mg/ml RNase (Qiagen, Valencia, CA, USA), and stained with 0.5 mg/ml propidium iodide (Sigma) in phosphate-buffered saline (PBS) on ice. Samples were analyzed by a flow cytometer (FACScan, Becton-Dickinson, Franklin Lakes, NJ, USA) for DNA content using propidium iodide. Ten thousand events were recorded and the proportion of cells in various phases of the cell cycle was analyzed with the Mod Fit Ltd. DNA analysis software program and Cell Quest (Becton-Dickinson). Three independent experiments were performed for each data set.

**Cell death detection by immunocytochemistry.** To confirm apoptotic cell death induced by PSK, the In Situ Cell Death

Detection kit (Roche, Lewes, UK), an immunocytochemical method to identify free 3'-OH ends localized in apoptotic bodies, was used. Direct fluorescence cytochemistry for TUNEL staining was performed using fluorescein TdT. Cells were incubated in the absence or presence of PSK (100  $\mu$ g/ml) in 6-well plates until 50% confluence. Media were aspirated from plates, and cells were washed twice with PBS and fixed with 4% paraformaldehyde solubilized in 0.1% Triton X-100 in 0.1% sodium citrate for 60 min at room temperature. Cells were washed twice with PBS and incubated with TUNEL reaction mixture for 60 min at 37°C in 5% CO<sub>2</sub>. Cells were again washed twice with PBS and analyzed by fluorescence microscopy (IX70, Olympus, Tokyo, Japan).

**Assay for STAT3 phosphorylation.** STAT3 phosphorylation was analyzed using a Cellular Activation of Signaling ELISA kit (CASE Kit: SuperArray Biosciences, Frederick, MD) according to the manufacturer's instructions with some minor modifications. Cells (1.5x10<sup>4</sup> cells) were seeded in 96-well plates, cultured for 24 h, and were treated with PSK (0, 10, 100  $\mu$ g/ml) for 60 min. Cells were then fixed with 4% formaldehyde for 20 min and processed for ELISA measurements with anti-pY705-STAT3 or anti-STAT3 antibodies. The bound antibodies were then detected with developing solution. Plates were analyzed using an ELISA reader at a wavelength of 450 nm. After washing twice with washing buffer, plates were treated with cell staining buffer, and read in an ELISA reader at a wavelength of 595 nm. Readings were taken to assess the total number of cells per well and these data were normalized for the number of cells in each well.

**Western blot analysis.** The protein expressions of Bax, Bcl-xL, Mcl-1, p-STAT3, and STAT3 were examined by western blot analysis, and equal protein loading was evaluated by GAPDH. Cultured cells treated with PSK (0, 10, 100  $\mu$ g/ml) for 48 h were collected and lysed with a lysis buffer for 60 min at 4°C, and debris was eliminated by centrifugation at 15000 rpm for 30 min at 4°C. The supernatant was collected and the protein concentration of each sample was measured by a protein assay rapid kit (Wako, Osaka, Japan). Equal amounts of protein (50  $\mu$ g) were mixed with SDS sample buffer containing 2-mercaptoethanol, boiled for 5 min, and loaded on 10% SDS-PAGE gels. It was transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare, Milwaukee, WI, USA) with a tank transfer system (Bio-Rad). Membranes for the antibody reaction of Bax, Bcl-xL, Mcl-1, STAT3, and GAPDH were blocked in 5% skimmed milk in TBS-T at room temperature for 1 h. Membranes for the antibody reaction of p-STAT3 were blocked in Blocking One-P (Nacalai Tesque) designed for applications with phospho-specific antibodies. Membranes were reacted with specific primary antibodies diluted as follows: Bax was 1:500, Bcl-xL was 1:1000, Mcl-1 was 1:1000, p-STAT3 was 1:1000, STAT3 was 1:500, and GAPDH was 1:500 at room temperature for 1 h. The bound primary antibody was detected by either anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (HRP). Signals were detected with ECL and western blotting detection reagents (GE Healthcare UK Limited, UK).

**Statistical analysis.** Data were reported as the mean  $\pm$  standard deviation of triplicate experiments. A Student's t-test was used

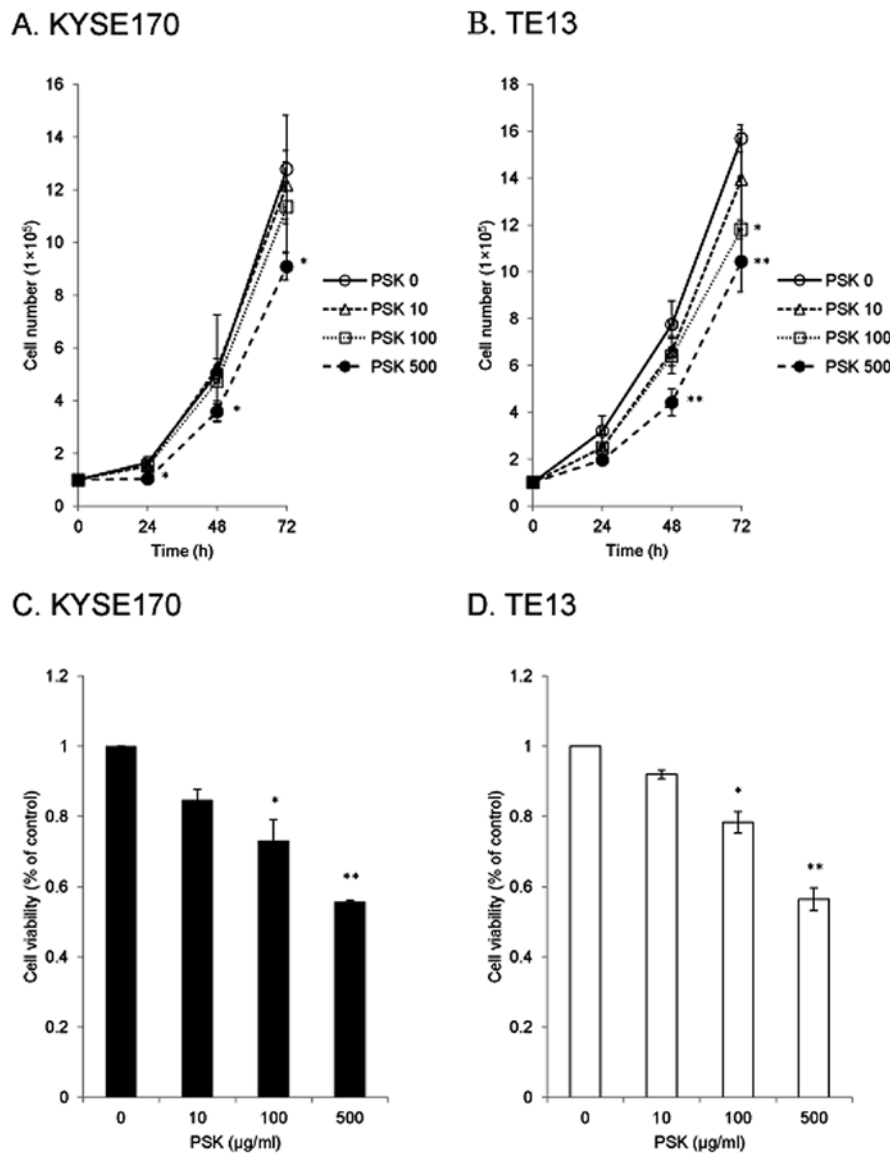


Figure 1. Effect of PSK on cell proliferation and viability. KYSE170 and TE13 cells were incubated with PSK at the indicated concentrations. Cell proliferation was assessed by cell counting. Cell viability was assessed by a WST-8 assay (\* $p<0.05$ , \*\* $p<0.01$ ).

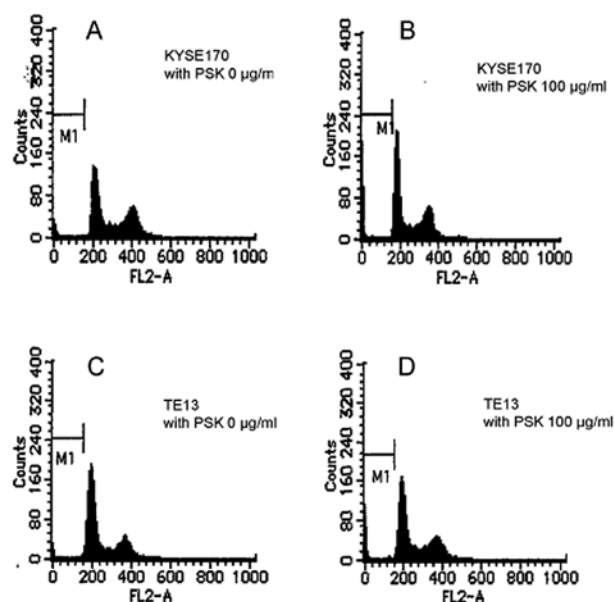
for statistical comparisons.  $p<0.05$  was considered significant. \* $p<0.05$  vs. control. \*\* $p<0.01$  vs. control.

## Results

**Effect of PSK on cell growth and viability.** In the cell growth assay, the cell growth ability of both cell lines was suppressed dose-dependently by PSK. In KYSE170 cells, the cell numbers at doses of 100 and 500  $\mu\text{g/ml}$  of PSK after 72-h incubation was 88.8% and 71.1% of the control, respectively (Fig. 1A). A significant difference was observed between the control and PSK-treated groups (500  $\mu\text{g/ml}$ ,  $p<0.05$ ). In TE13 cells, the cell numbers at doses of 10, 100, and 500  $\mu\text{g/ml}$  of PSK after 72-h incubation was 89.0%, 75.3%, and 66.5% of the control, respectively (Fig. 1B). Significant differences were observed at 100 and 500  $\mu\text{g/ml}$  of PSK ( $p<0.05$ , 0.01, respectively). Similarly, in the WST-8 assay, cell viability was suppressed dose-dependently by PSK. In KYSE170 cells, cell viability at doses of 10, 100, and 500  $\mu\text{g/ml}$  of PSK was  $84.7\pm2.9$ ,  $73.1\pm5.9$ , and  $55.6\pm0.5\%$

of control, respectively (Fig. 1C). In TE13 cells, cell viability at doses of 10, 100, and 500  $\mu\text{g/ml}$  of PSK was  $91.9\pm1.2\%$ ,  $78.2\pm3.0\%$ , and  $56.4\pm3.2\%$  of the control, respectively (Fig. 1D). In both cell lines, significant differences were observed between control and PSK-treated cells at 100 and 500  $\mu\text{g/ml}$  of PSK ( $p<0.05$ , 0.01, respectively).

**Detection of apoptosis induced by PSK.** Apoptotic cell death induced by PSK was evaluated by cell cycle analysis and immunocytochemistry. In cell cycle distribution, the proportion of sub-G1 cells, which indicates the apoptotic cell population, was 3.06% and 1.31% in the absence of PSK in KYSE170 and TE13, respectively, whereas the proportion increased to 5.21% and 4.14% in the presence of PSK (100  $\mu\text{g/ml}$ ) in KYSE170 and TE13, respectively (Fig. 2). In addition, PSK-induced apoptotic cells were morphologically detected by phase contrast microscopy and immunocytochemically detected as TUNEL-positive cells under fluorescence microscopic observation (Fig. 3).



Cell line	PSK (100 µg/ml)	sub G1 (%)	G1 (%)	S (%)	G2/M (%)
KYSE170	—	3.06	40.15	35.96	23.90
	+	5.21	44.26	36.13	19.61
TE13	—	1.31	60.05	24.55	15.40
	+	4.14	46.68	33.33	19.99

Figure 2. Effect of PSK on cell cycle distribution and apoptosis. KYSE170 and TE13 cells treated with 100 µg/ml of PSK were analyzed by flow cytometry to assess cell cycle distribution and apoptosis. The M1 fraction indicates the sub-G1 apoptotic cell population. The percentage of each cell cycle population (sub-G1, G1, S, and G2-M) are shown in (E).

**Effect of PSK on STAT3 phosphorylation.** When evaluated by ELISA, STAT3 was constitutively phosphorylated in both cell lines. The total amount of STAT3 was not affected by PSK, whereas phosphorylated STAT3 was decreased by PSK in a dose-dependent manner (Fig. 4A and B). Similarly, when evaluated by western blot analysis, p-STAT3 expression was decreased by PSK treatment in a dose-dependent manner, although STAT3 expression was not affected by PSK treatment (Fig. 4C).

**Effect of PSK on Bcl-2 family protein expression.** The effect of PSK on the expression of Bcl-2 family proteins (anti-apoptotic Bcl-xL and Mcl-1, and pro-apoptotic Bax) was investigated by western blot analysis. In both cell lines, Bax expression was increased by PSK in a dose-dependent manner, whereas Bcl-xL and Mcl-1 expression was not affected by PSK (Fig. 5).

## Discussion

PSK, an orally administered biological response modifier, has been shown to be more effective in prolonging the survival of

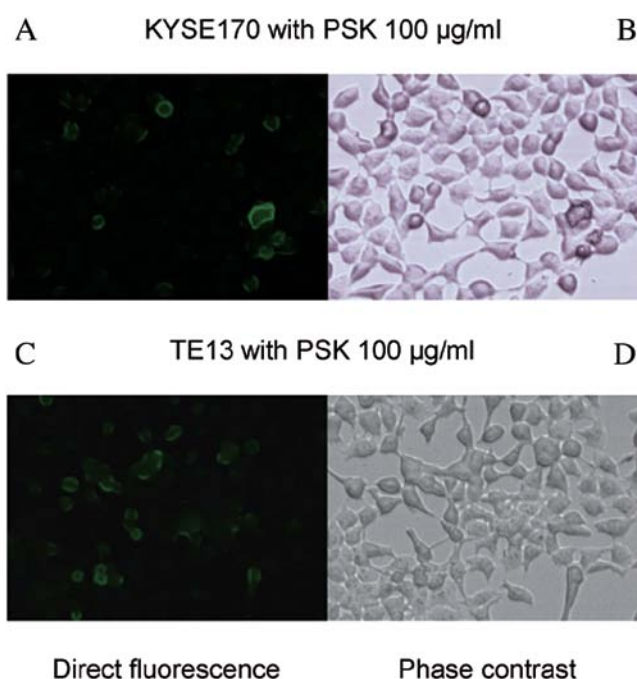


Figure 3. Immunocytochemical detection of PSK-induced apoptotic cells. KYSE170 and TE13 cells were treated with 100 µg/ml of PSK. Cells were then treated for the fluorescent detection of apoptotic cells according to the method described in Materials and methods. Fluorescence (A and C) and phase-contrast (B and D) views of the identical microscopic field of KYSE170 (A and B) and TE13 (C and D) cells are shown.

patients with gastric cancer or colorectal cancer after a curative resection by its combination use with chemotherapy compared with chemotherapy alone (21,22). The anti-tumor effects of PSK have been analyzed, with a focus mainly on its action on the host's immune response such as cytokine induction in human peripheral blood mononuclear cells, enhancement of the cytotoxicity of human tumor infiltrating lymphocytes, and inhibition of plasma TGF-β levels in tumor-bearing mice (23-25). On the other hand, there are several studies on the direct action of PSK on tumor cells; MMP inhibition and suppression of invasion in gastric carcinoma cells; G1 arrest in gastric carcinoma cells; enhancement of the expression of major histocompatibility complex (HLA) class I; and apoptosis induction in leukemia and lymphoma cells (26-30). The present study demonstrated that PSK suppressed cell proliferation and induced apoptosis in esophageal carcinoma cells, and inhibition of activated STAT3 and induction of pro-apoptotic Bax played a role in apoptosis induction.

With regard to the causal relationship between STAT3 inhibition and apoptosis, STAT3 inhibition by a selective STAT3 inhibitor has been shown to induce apoptosis in various carcinoma cells (31-33). Nielsen *et al* reported that inhibition of STAT3 phosphorylation by a JAK kinase inhibitor increased Bax expression, decreased Bcl-2 expression, and induced apoptosis in mycosis fungoides tumor cells (33). This suggests that PSK-mediated inhibition of STAT3 may regulate the expression of Bcl-2 family proteins. Bcl-2 family proteins consist of anti-apoptotic molecules such as Bcl-2, Bcl-xL, and Mcl-1, and pro-apoptotic molecules, such as Bax, Bad, and Bak, and the expression ratio of pro-apoptotic to anti-apoptotic proteins is

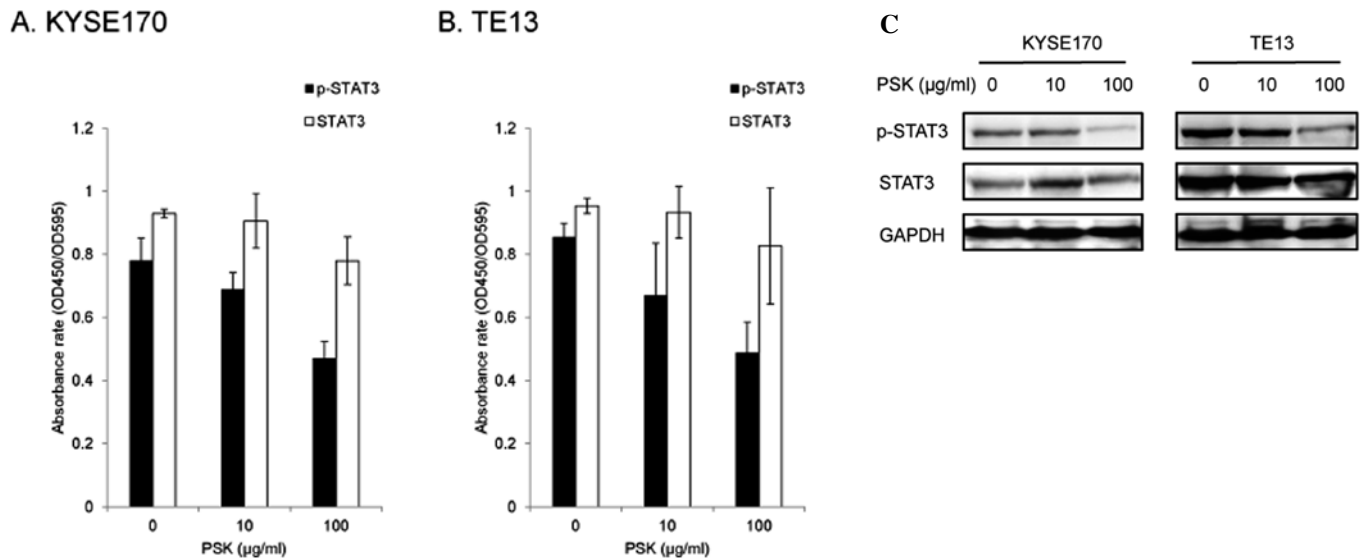


Figure 4. Effect of PSK on STAT3 expression. KYSE170 and TE13 cells were treated with PSK at the indicated concentrations. Total and phosphorylated STAT3 expressions were then assessed by an ELISA kit for STAT3 Y705 (A and B) and western blotting (C). GAPDH was used as an internal control.

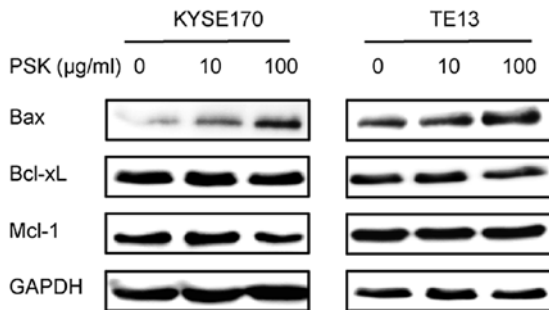


Figure 5. Effect of PSK on Bcl-2 family protein expression. KYSE170 and TE13 cells were treated with PSK at the indicated concentrations. Bax, Bcl-xL, and Mcl-1 expressions were then assessed by western blotting. GAPDH was used as an internal control.

crucial to determine survival or death following an apoptotic stimulus (34). Ye *et al* reported that Grifolin isolated from a mushroom (*Albatrellus confluens*) increased the expression ratio of Bax to Bcl-2 and induced apoptosis in a human nasopharyngeal carcinoma cell line (35). In our study, PSK, a protein-bound polysaccharide isolated from the *Coriolaceae* mushroom, upregulated Bax expression without any substantial change in Bcl-xL or Mcl-1 expression, and induced apoptosis in esophageal carcinoma cell lines with a subsequent increase in the ratio of pro-apoptotic to anti-apoptotic proteins. PSK is considered to be a multi-molecular target drug according to its molecular structure. This suggests that PSK may act independently on STAT3 and Bcl-2 family proteins. PSK-mediated actions on STAT3 and Bcl-2 family proteins may collectively determine the expression status of Bcl-2 family proteins.

In conclusion, the present study demonstrated that PSK inhibited cell proliferation and induced apoptosis in esophageal carcinoma cell lines through STAT3 inhibition and Bax augmentation. PSK exerts dual antitumor effects by not only stimulating the host's immune response, but also acting on tumor cells, which is a unique characteristic distinct from recent molecular target drugs. Blocking STAT3 signaling in tumor

cells has been shown to induce apoptosis, inhibit cell proliferation, suppress angiogenesis, and stimulate immune responses (36-38). Accordingly, our present study on PSK-mediated anti-tumor effects focusing on STAT3 signaling will provide useful information for the introduction of molecular target therapy into conventional treatment modalities for esophageal cancer.

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