**Inhibitory Effect of Astragalus Polysaccharide Combined with Cisplatin on Cell Cycle and Migration of Nasopharyngeal Carcinoma Cell Lines**

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**Background** Astragalus polysaccharide (APS) had shown great promise in anti-tumour activities in our previous studies. The present study was designed to investigate whether APS has synergistic effect with cisplatin on the growth-inhibitory of human nasopharyngeal carcinoma cell lines and the possible mechanism.

**Methods** Here, nasopharyngeal carcinoma cell lines (CNE-1) were divided into CNE-1 group, Cisplatin treatment group (2 µg/mL Cisplatin), APS treatment group (200 µg/mL APS) and combination group (2 µg/mL Cisplatin and 200 µg/mL APS). The proliferation inhibition rate of CNE-1 cells was determined by Cell Counting Kit-8 (CCK-8) method after treatment with different concentrations of APS for 24, 48, and 72 h. Apoptosis rates and cell cycle retardation of cells were detected by flow cytometry. Cell migration and invasion was evaluated by transwell assay. Western blotting and quantitative (q)RT-PCR were performed to detect the expression of Bcl-2, Bax, caspase-3, matrix metalloproteinase-2 (MMP-2), p53 and matrix metalloproteinase-9 (MMP-9) proteins in CNE-1 cells. Results APS have an inhibition on the proliferation of CNE-1 cells with time and dose dependence manner. Both the APS and combination therapy could promote apoptosis of CNE-1 cells, with the count of cells increased in G0/G1 and S phase while decreased in G2/M phase, and inhibited the migration and invasion of CNE-1 cells. Moreover, co-administration of Cisplatin and APS was more efficacious for the antitumor effect than either agent alone, as evidenced by the significant decrease in MMP-9 level and increase in p53. Conclusion APS, in combination with cisplatin, had significantly synergistic growth-inhibitory effect on nasopharyngeal carcinoma cell lines, which may be related to cell cycle and migration induction.

**Key words** Astragalus polysaccharide; cisplatin; combination therapy; nasopharyngeal carcinoma cell line

**INTRODUCTION**

As a malignant tumor occurring at the top of nasopharyngeal cavity and lateral wall, nasopharyngeal carcinoma incidence frequently in southeast Asia, northern Africa, some Mediterranean coastal countries and south China, especially in Guangdong and Guangxi areas. 1) Local recurrence and distant metastasis are the main causes of treatment failure for nasopharyngeal carcinoma. Though concurrent chemoradiotherapy like cisplatin-based chemotherapy can solve this problem, the living quality and therapy compliance of tumor patient were unsatisfactory for a series of adverse reactions such as gastrointestinal reactions and renal toxicity. 2)

Combination therapy has achieved phased efficacy in tumor therapy, preliminary studies shows that Astragalus polysaccharide (APS), as one of main active constituent in Astragalus, had remarkably effect in immunity enhancement and antitumor. 3) A large number of in vitro and in vivo experiments have found that APS has strong anti-tumor activity, which through recruiting cytokines capable of killing tumor cells or regulating the immune system to destroy the cancers. Moreover, APS could induce apoptosis of tumor cells by promoting cell differentiate to G0-G1 and G2-M phases. 5) Recent research showed that combination therapy of APS and cisplatin presented cytotoxicity more effectively than them using alone in Cervical cancer, 6) lung cancer, 7) and other multiple solid tumor cells. 9) And APS also could reduce the bone marrow suppression and toxicity induced by chemotherapy in mice. 9) So, we speculate that APS could as the subsidiary medicine of chemotherapy in nasopharyngeal carcinoma. However, the mechanism under this activity was unclear.

In this study, we investigated the effect and mechanism of APS combined with cisplatin on proliferation, apoptosis, migration and invasion of nasopharyngeal carcinoma cell lines (CNE-1) and preliminary analysis the underlying mechanism, in order to promote clinical application of combination therapy of APS and cisplatin treatment and prevent recurrence in nasopharyngeal carcinoma.

**MATERIALS AND METHODS**

**Cell Culture and Proliferation Assay** The CNE-1 cell which ordered from Shanghai Cell Bank, Culture preservation Committee, Chinese Academy of Sciences (China), was cultured in formulated RPMI-1640 Medium (Gibco, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37°C in a humidified incubator with an atmosphere containing 5% CO2. The culture medium was changed daily. Cell proliferation was assessed by the Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Japan) assay to measure the cytotoxic response to APS. In brief, 200 µL per well of CNE-1 suspension was cultured in 96-well plates for 24, 48, and 72 h after treatment with each final concentration of APS (Solarbio, A7970) in 50, 100, 200, and 400 µg/mL, and the samples were assessed following the conventional procedure. The optical

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density (OD) was measured at 450 nm. Cell viability was calculated as the following ration: cell viability = OD\text{sample} / OD\text{control} \times 100%.

**Annexin V-Fluorescein Isothiocyanate (FITC)/Propidium Iodide (PI) Apoptosis and Cell Cycle Assay** After 48 h treatment, apoptotic rates were analyzed by flow cytometry using Annexin V-FITC/PI apoptosis detection kit (KeyGen, Nanjing, China). Staining was performed according to the manufacturer’s instruction, and flow cytometry was conducted on a CytoFLEX flow cytometer (Beckman Coulter, U.S.A.). Counted the populations of Annexin V⁺/PI⁻ (early apoptotic cells) and Annexin V⁺/PI⁺ (late apoptotic cells). For cell cycle assay, we fixed the cell at 4°C with 1 mL 75% ethanol precooled at −20°C for 1 h, after concentration adjustment. Washing twice with 1 mL phosphate buffered saline (PBS) to remove the supernatant, before mixed with 300 µL (PI) in the dark at 4°C for 20 min. Afterwards, a flow cytometer was employed to record the red fluorescence at excitation wavelength of 488 nm.

**Transwell Assay** The 200 µL cell resuspended with RPMI Fig. 1. Effect of APS at Different Concentration and Treatment Time on Proliferation Inhibition Rate of CNE-1 Cells

![Fig. 1](image_url)

Fig. 2. Effect of APS and/or Cisplatin on the Invasion and Metastasis of CNE-1
A. Quantitative analysis of cell metastasis by biological image analysis; B. Quantitative analysis of cell invasion by biological image analysis system. Data are presented as mean ± standard deviation (S.D.) (error bar). *p < 0.05, **p < 0.01, ***p < 0.001, vs. CNE-1 group, #p < 0.05, vs. combination group.
I640 medium with cell density adjusted to $1 \times 10^5$ cells/mL, was added into the apical chamber of the Transwell chamber covered with Matrigel (BD Biosciences, U.S.A.) and added 600 µL cell supernatant containing 10% FBS to the basolateral chamber. After conventional culture for 48 h, cells were fixed with 4% paraformaldehyde for 15 min, and stained with 0.5% crystal violet solution (formulated with methanol) for 10 min. Five fields ($200 \times$) were randomly selected and photographed using an inverted DMi8 microscope (Leica, Germany). The transmembrane cells were counted. Each experiment group was set with 3 duplicated wells.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blots** Cell lysates were prepared in ethylene diamine tetra acetic acid-freeley buffer containing complete protease inhibitors and were centrifuged at 12000 × g for 10 min. We performed 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis to separate the cell protein sample and then transferred to polyvinylidene fluoride membranes, blocked with Tris buffered saline-Tween 20 containing 5% skim milk. After incubated with primary antibodies (1:1000 dilution), the membranes were followed by incubation with secondary antibodies (1:3000 dilutions) for 1 h at room temperature. The blots were developed using an ECL chemiluminescence kit (Millipore, U.S.A.), and quantified the gray value in each blot by using Image J software. All the antibody purchased from CST (U.S.A.).

**RNA Extraction and Quantitative RT PCR** Total RNA in the CNE-1 cells were isolated using the RNAiso Plus (TaKaRa, Japan) according to the manufacturer’s instructions. For assessing the expression of Bcl-2, caspase-3, p53, matrix metalloproteinase-9 (MMP-9) and $\beta$-actin, 1 µg RNA was reverse-transcribed using Superscript III and random primers (TaKaRa). cDNA samples were amplified in a CFX96 Real-time System (Bio-Rad Laboratories, U.S.A.) using the SYBR Green Master Mix (TaKaRa) and specific primers according to the manufacturer’s instructions. All primers were purchased from TaKaRa. The primer sequences used are the following: Hsa-MMP9-F: GAC GCC ATC GCG GAG ATT G, Hsa-MMP9-R: GCC ACT TGT CGG CGA TAA G; Hsa-p53-F: CAA GAC AGA AGG GCC TGA CTC, Hsa-p53-R: CAT CTC CCA AAC ATC CCT CAC; Hsa-Caspase3-F: GGA TACT GGG TGC AGT CAT TAT G, Hsa-Caspase3-R: GGTT TGG AGC CTT TGAC CTA G; Hsa-Bcl2-F: CGG GGA GAT GTG GAT GAA GTAC, Hsa-Bel2-R: GTG GCC GTT CAT GTA CTC AG; Hsa-$\beta$-Actin-F: GCC ACC CAG CAC AAT GAA G, Hsa-$\beta$-Actin-R: GGT GTA ACG CAA CTA AGT CAT AG. Fold changes of mRNA quantity among groups were determined by the $\Delta$CT method. The data were normalized using $\beta$-actin expression as the reference.

**Statistical Analysis** All data were processed using SPSS 19.0 statistical software (IBM Corp., Armonk, NY, U.S.A.) and expressed as mean ± standard deviation (S.D.). The $t$-test was used to compare two groups while one-way ANOVA was used for multiple group comparison. $p < 0.05$ indicated a statistically significant value.

**RESULTS**

**APS Inhibited the Proliferation of CNE-1 Cells with Dose and Time Dependent** We assessed the cytotoxicity of APS to CNE-1 by performing CCK-8 assays. The results confirmed that treatment with APS at concentrations of 50,
100, 200, and 400 μg/mL, respectively after 24, 48, and 72 h, inhibited the proliferation of CNE-1 obviously (Fig. 1) Thus, APS was used at concentrations of 200 μg/mL with treatment 48 h during the follow-up experiments.

**Effects of APS Combined with Cisplatin on the Invasion and Metastasis of CNE-1** Recent research revealed that combination therapy of APS and cisplatin presented cytotoxicity more effectively than those using alone in the treatment of cancer. So, we detected the invasion and metastasis of CNE-1 after treated with different drugs, showing that whatever in cisplatin, APS or combination treatment group, the invasion and metastasis of CNE-1 were suppressed (Fig. 2). Interestingly, combination treatment group showed a better curative effect than other in metastasis test.

**Combination Therapy of APS and Cisplatin Inhibited the Cell Cycle and Promoted the Apoptosis of CNE-1** To examine the effect of combination therapy of APS and cisplatin on the cell cycle and apoptosis of CNE-1, we treated the cell with drugs for 48 h, which has been shown to blocking the cells in the G0/G1 and S phases (Fig. 3A) and the apoptosis rates of CNE-1 in combination group was higher than the other group (Fig. 3B).

**Effect of Combination Therapy of APS and Cisplatin on the Expression of Oncogene Genes and Associated Protein** To further confirm the inhibiting effect of combination therapy on CNE-1, we performed the quantitative (q)RT-PCR and Western blots assays to examined the expression of oncogene genes and associated protein. The qRT-PCR results showed that the combination therapy enhanced the effect in down-regulation of MMP-9 gene expression (Fig. 4a) and improving p53 (Fig. 4b), except the Bcl-2 (Fig. 4c) and caspase-3 (Fig. 4d) in CNE-1. Therefore, we detected associated protein in CNE-1, finding that the results of MMP-9 (Fig. 5a) and p53 (Fig. 5e) were consistent with qRT-PCR. However, for the Bcl-2 (Fig. 5c) or caspase-3 (Fig. 5d), there were no synergistic effect in combination group, which meant that the pro-apoptotic of combination therapy depended on other mechanisms. And there was no obvious change of MMP-2 (Fig. 5b).

**DISCUSSION**

Nasopharyngeal carcinoma (NPC), a kind of malignant tumor with high invasive and high metastasis rate in nasopharyngeal cavity. Current research performed that the abnormal processes of differentiation and growth or cell cycle regulation associated with unlimited proliferation of tumor cells closely. In this study, we verify primarily the anti-tumour activity of APS though CCK8 assay, in which shown APS inhibited the proliferation of CNE-1 with dose and time dependent. Then, our study performed the transwell assay to investigate whether APS has synergistic effect with cisplatin on cell migration and invasion. MMPs degraded and reshaped the extracellular matrix and basement membrane by proteolysis, thus promoting tumor invasion and metastasis.

Previous researches demonstrated that MMP-2(10) and MMP-9(11) destroyed basement membrane, promoting naso-
pharyngeal carcinoma cell break through tissue boundaries and colonize lymph gland tissues. The results demonstrated that APS combine with cisplatin performed a better curative effect than other in metastasis test, which confirmed by the down-regulated expression of MMP-9 in CNE-1. However, there was no significant change in the expression level of MMP-2 protein, indicating that APS combined with cisplatin may regulate the activity of collagen and affect the activation of other related substrates such as growth factors and inhibit the migration and invasion of nasopharyngeal carcinoma cells mainly through MMP-9 rather than MMP-2.

Clinical research found that the expression of p53 was positively correlated with MMP-9 in solid tumors. Furthermore, p53 protein played an important role in the invasion and metastasis of nasopharyngeal carcinoma. Wild-type p53 is an important tumor suppressor gene in vivo, which is mainly involved in DNA repair and replication. Our study showed the APS combine with cisplatin would enhance the effect in blocking the cell cycles in the G0/G1 and S phases and improving expression of p53. For then, we investigate whether APS has synergistic effect with cisplatin on the apoptosis of CNE-1. The flow cytometry results shown that the combination treatment will promote the apoptosis of CNE-1. Researchers have found that upregulation of Bcl-2 protein overexpression would inhibition the apoptosis of nasopharyngeal carcinoma cells. Bcl-2 protein also can regulate caspase-3 and downstream protease cascade reactions by affecting cytochrome c, apoptotic protease activators apAF-1 and caspase-9 in the cytoplasmic, so as to inhibit cancer cell apoptosis. So our research detected the expression of Bcl-2 and caspase-3 protein, finding that the expression level of apoptosis inhibitor Bcl-2 in CEN-1 was significantly decreased, and the caspase-3, one of the important effectors of apoptosis, was on opposite. However, there was not a synergistic effect for the Bcl-2 or caspase-3 expression in combination group, which meant that the pro-apoptotic of combination therapy depended on other mechanisms. The further study would be needed to confirm it.

In conclusion, the results obtained in the present study indicate that APS in combination with cisplatin results in greater growth inhibition and apoptosis induction than did either agent alone on CNE-1, which may be related with down-regulation of MMP-9, up-regulation of p53 and this combination might be useful for nasopharyngeal carcinoma treatment.

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Conflict of Interest The authors declare no conflict of interest.

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