

# JNK pathway inhibition enhances chemotherapeutic sensitivity to Adriamycin in nasopharyngeal carcinoma cells

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**Abstract.** The role of c-Jun N-terminal kinases (JNKs) in the pathogenesis of cancer is well-known due to their involvement in carcinogenesis. Although previous studies have discussed different functions of JNKs depending on cell type, the present study aimed to investigate the function of JNKs in nasopharyngeal carcinoma (NPC) cells, as well as their involvement in chemotherapy sensitivity to Adriamycin. The present results showed that Adriamycin administration reduced cell viability and led to elevated expressions of c-Jun, phosphorylated JNK and phosphorylated c-Jun, indicating an activated JNK pathway. Notably, JNK inhibition by SP600125 also reduced cell growth. Thus, Adriamycin treatment combined with SP600125 was more effective on cell growth inhibition than each agent alone. The apoptosis analysis confirmed the reduction in cell growth. Therefore, these data provide evidence that the JNK pathway activity is negatively associated with cell viability, and its decline could sensitize NPC cells to Adriamycin.

## Introduction

Nasopharyngeal carcinoma (NPC) is a squamous cell carcinoma caused by a combination of factors, such as viral, dietary and genetic factors (1). It is widely diffused in Southern China and Southeast Asia and it is notorious for its local invasion and distant metastasis at the time of diagnosis (2).

Chemotherapy and radiotherapy are the main strategies to treat NPC patients (3). However, poor prognosis and therapeutic failure are the most common outcomes subsequent to treatment of NPC at an advanced stage, even with concurrent chemoradiotherapy (4). Therefore, chemosensitization or radiosensitization is essential for the successful treatment of NPC.

Treatment with chemotherapeutic drugs activates several biochemical signaling pathways, including the c-Jun N-terminal kinase (JNK) pathway (5). JNKs are serine/threonine kinases that belong to the mitogen-activated protein kinase family (6), and are responsive to diverse stress (7,8). When phosphorylated by upstream kinases, JNK in turn phosphorylates and activates three nuclear transcription factors, consisting of c-Jun, activating transcription factor 2 and Elk-1 (9-11), which regulate several important cellular functions, including cell growth, differentiation, survival and apoptosis.

Previous studies have revealed the conflicting roles of JNK, depending on tumor type (12). JNK pathway inactivation decreases tumor growth in gastric or intestinal tumors (13,14), indicating its pro-tumorigenic role. By contrast, JNK pathway inactivation promotes tumor development in breast cancer (15,16), while JNK activation can reduce cell proliferation and maintain epithelial differentiation in non-small cell lung cancer (NSCLC) cells (17), suggesting its anti-tumorigenic role. The JNK pathway also mediates opposite functions on apoptosis in a tumor type-dependent manner, such as a pro-apoptotic role in human gastric cancer cells (18) vs. an anti-apoptotic role in SCLC cells (19).

The present study explored the role of the JNK pathway in NPC 5-8F cell line, as well as its influence on chemotherapeutic sensitivity to Adriamycin. The present results may suggest that the members of the JNK pathway could represent valid therapeutic targets on NPC treatment.

## Materials and methods

**Cell culture.** 5-8F cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (GE Healthcare Life

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Sciences, Chalfont, UK), 100 U/ml penicillin and 100  $\mu\text{g/ml}$  streptomycin (Beyotime Institute of Biotechnology, Haimen, China) at 37°C in a 5%  $\text{CO}_2$  humidified atmosphere.

**MTT assay.** Briefly, cells were seeded at a density of  $0.6 \times 10^4$  cells per well in 100  $\mu\text{l}$  RPMI-1640 medium in a 96-well plate. Subsequent to incubation with different concentrations of Adriamycin (0.1, 0.5, 1, 5 and 10  $\mu\text{g/ml}$ ; Zhejiang Hisun Pharmaceutical Co. Ltd, Taizhou, China) and the JNK inhibitor SP600125 (2.5, 5, 10, 20 and 40  $\mu\text{M}$ ; Beyotime Institute of Biotechnology, Beijing, China) for 24 h, MTT assay was performed by the MTT Cell Proliferation and Cytotoxicity Assay Kit (Beyotime Institute of Biotechnology, Beijing, China). The absorbance was measured at 490 nm using aN iMark Spectramax microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Western blot analysis.** Total proteins from treated or untreated cells were extracted by radioimmunoprecipitation buffer with protease inhibitor (Beyotime Institute of Biotechnology), and separated by electrophoresis in SDS-PAGE gels. Next, proteins were transferred to polyvinylidene difluoride membranes (GE Healthcare Life Sciences), followed by immunoblotting with 5% milk and incubation with the following antibodies: Phosphorylated JNK (dilution, 1:1,000; catalog no., 4668; Cell Signaling Technology, Inc., Danvers, MA, USA); c-Jun (dilution, 1:1,000; catalog no., 9165; Cell Signaling Technology, Inc.); phosphorylated c-Jun (dilution, 1:1,000; catalog no., 9164; Cell Signaling Technology, Inc.); and GAPDH (dilution 1:2,000; catalog no., 2118; Cell Signaling Technology, Inc.). The next day, membranes were washed with TBST and incubated with secondary antibodies (dilution, 1:3,000; catalog no., SA00001-2; ProteinTech, Chicago, IL, USA) for 1-2 h. Subsequent to washing with TBST, an electrochemiluminescence Luminata™ Crescendo Western horseradish peroxidase substrate (catalog no., WBLUR0100; EMD Millipore, Billerica, Ma, USA) was performed (GE Healthcare Life Sciences) for the detection of immunoblotting according to the protocol of the manufacturer.

**TUNEL assay.** Cells were seeded onto a 24-well plate ( $2 \times 10^4$  cells in 0.5 ml medium per well), and treated with Adriamycin (0.1  $\mu\text{g/ml}$ ) in combination with SP600125 (2.5  $\mu\text{M}$ ), as well as each agent alone. Twenty-four h later, TUNEL assay was performed using an *In Situ* Cell Death Detection kit (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. In total, three fields per slice were randomly selected and analyzed, and each experiment was repeated three times. The apoptotic index was calculated as the ratio of the TUNEL positive cell number to the total cell number in each field.

**Flow cytometry.** Cells were seeded onto 6-well plate ( $1.5 \times 10^4$  cells in 2 ml medium per well), and treated with Adriamycin (0.5  $\mu\text{g/ml}$ ) in combination with SP600125 (10  $\mu\text{M}$ ), as well as each agent alone. Twenty-four h later, cells were collected and stained for Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 15 min at room temperature, according to the

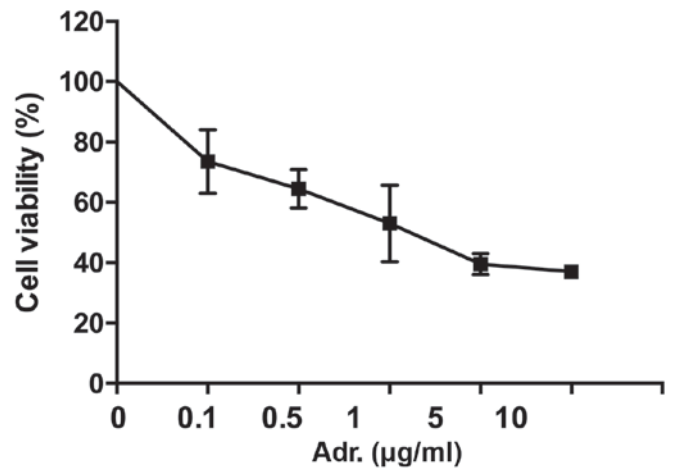


Figure 1. Adr. reduced nasopharyngeal carcinoma cell viability. 5-8F cells were treated with increasing concentrations of Adr. (0-10  $\mu\text{g/ml}$ ) for 24 h, and then cell viability was assessed by MTT assay. Data are expressed as the mean  $\pm$  standard deviation of at least triplicate experiments. Adr., Adriamycin.

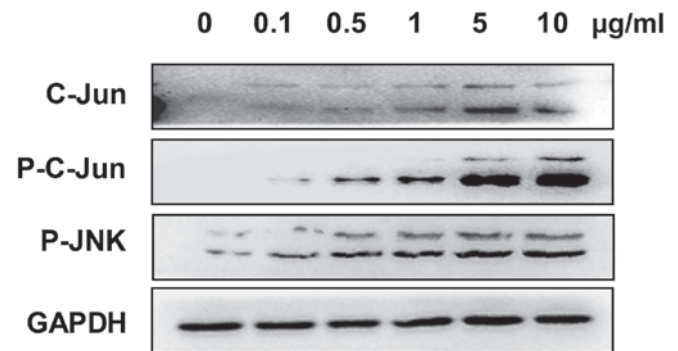


Figure 2. Adriamycin activated the JNK signaling pathway in nasopharyngeal cells. 5-8F cells were treated with increasing concentrations of Adriamycin (0-10  $\mu\text{g/ml}$ ) for 24 h, and then expression levels of members in JNK pathway were analyzed by western blot analysis. P-, phosphorylated; JNK, c-Jun N-terminal kinase.

manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were subsequently analyzed by flow cytometry (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA).

**Statistical analysis.** Data were analyzed by SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA), and expressed as the mean  $\pm$  standard deviation. Statistical analysis was performed using paired Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Adriamycin caused dose-dependent cytotoxicity on NPC cells.** The effect of Adriamycin on NPC cells was evaluated by MTT assay (Fig. 1). 5-8F cells were treated with different concentrations of Adriamycin. Twenty-four h later, the cell growth was inhibited, with this being statistically significant in cells treated with  $\geq 0.1$   $\mu\text{g/ml}$  Adriamycin ( $P < 0.05$ ). These

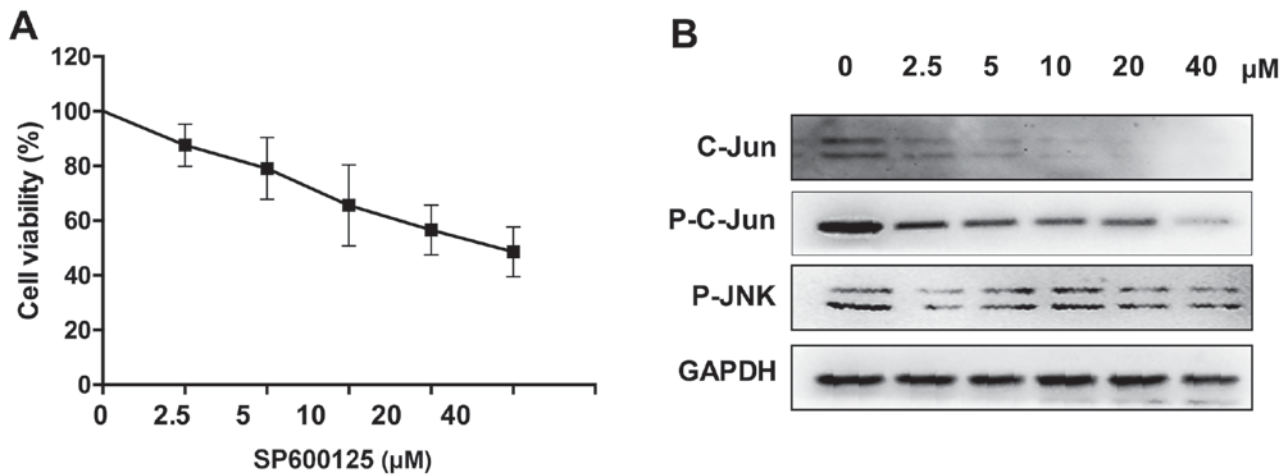


Figure 3. JNK pathway inhibition reduced NPC cells viability. 5-8F cells were treated with increasing concentrations of SP600125 (0-40  $\mu$ M) for 24 h. (A) Cell viability was assessed by MTT assay. Data are expressed as the mean  $\pm$  standard deviation of at least triplicate experiments. (B) Western blot analysis demonstrated high inhibition of the JNK pathway. P-, phosphorylated; JNK, c-Jun N-terminal kinase.

data indicated 5-8F cells were sensitive to Adriamycin and showed concentration-dependent sensibility.

*Adriamycin activated JNK signaling pathway in NPC cells.* The expression of c-Jun, phosphorylated-JNK and phosphorylated-c-Jun was analyzed to evaluate whether the JNK signaling pathway was involved in Adriamycin-associated cell inhibition in NPC cells. The present results showed an increased expression of these genes in 5-8F cells treated with Adriamycin, indicating an activation of the JNK pathway (Fig. 2).

*JNK signaling pathway inhibition caused cytotoxicity on NPC cells.* To investigate the effect of the JNK pathway on the viability of NPC cells, 5-8F cells were treated with the JNK pathway inhibitor SP600125 to block the signaling pathway. Western blot analysis demonstrated high inhibition efficiency (Fig. 3B). Following treatment for 24 h at a concentration of 5  $\mu$ M, the number of 5-8F cells was 79% ( $P<0.05$ ), indicating that JNK signaling pathway inhibition can reduce NPC cells growth (Fig. 3A).

*JNK pathway inhibition sensitized NPC cells to Adriamycin.* Since the aforementioned results revealed that Adriamycin was an effective agent against NPC cells and activated the JNK pathway, which was a growth promoter in NPC cells, it was examined whether JNK pathway inhibition enhanced chemosensitivity of NPC cells to Adriamycin. In the presence of 10  $\mu$ M SP600125, the toxic effect of Adriamycin was evidently increased by 15% for 5-8F cells ( $P<0.05$ ) compared with Adriamycin alone (Fig. 4). These results provide an indication that JNK pathway inhibition sensitized NPC cells to the cytotoxic effects of Adriamycin.

*Adriamycin combined with JNK pathway inhibition induced apoptosis in NPC cells.* To investigate the effect of Adriamycin and JNK pathway inhibition on apoptotic cell death, NPC cells treated with either agent or a combination of the two were stained with Annexin V FITC/PI and TUNEL,

which were analyzed by flow cytometry and fluorescent microscopy, respectively. As shown in Fig. 5, following treatment with low concentrations of Adriamycin (0.1  $\mu$ g/ml) or SP600125 (2.5  $\mu$ M) alone, the cellular apoptotic rate in 5-8F cells increased between 3.9 and 6.6% ( $P<0.05$ ), and 3.9 and 10.4% ( $P<0.01$ ), respectively, while the combination treatment increased between 3.9 and 14.1% ( $P<0.001$ ). The results were also confirmed by treatment with increased concentrations of Adriamycin (0.5  $\mu$ g/ml) or SP600125 (10  $\mu$ M), which were analyzed by flow cytometry (Fig. 5C). These data demonstrated increased efficiency in the apoptosis induction by Adriamycin, combined with JNK pathway inhibition.

## Discussion

Chemotherapy is the main strategy for cancer treatment, but drug resistance occurs to limit the efficacy in clinical use. Currently, the screening of agents inducing chemosensitivity is the main method to overcome this problem (20). In the present study, JNK pathway inhibition was shown to cause cytotoxicity on NPC cells, indicating that this signaling pathway exerts a growth promotion function in NPC cells. 5-8F cells treated with Adriamycin showed activated JNK signaling, which may resist Adriamycin-induced cell death. According to these findings, 5-8F cells treated with a combination of the JNK pathway inhibitor SP600125 at a fixed concentration and Adriamycin at increasing concentrations showed elevated cell death compared with cells treated with each agent alone.

In previous years, studies have revealed multiple complicated signaling networks in NPC growth inhibition (21,22). The present study found that the JNK pathway was activated 24 h subsequent to Adriamycin administration, which was consistent with a study reporting that the JNK pathway was sensitive to cytotoxic drugs (23).

Previous studies have described the conflicting roles of the JNK pathway in tumor growth (13,14). In the present study, 5-8F cells were treated with a JNK pathway inhibitor, resulting in significantly decreased cell viability and increased apoptotic



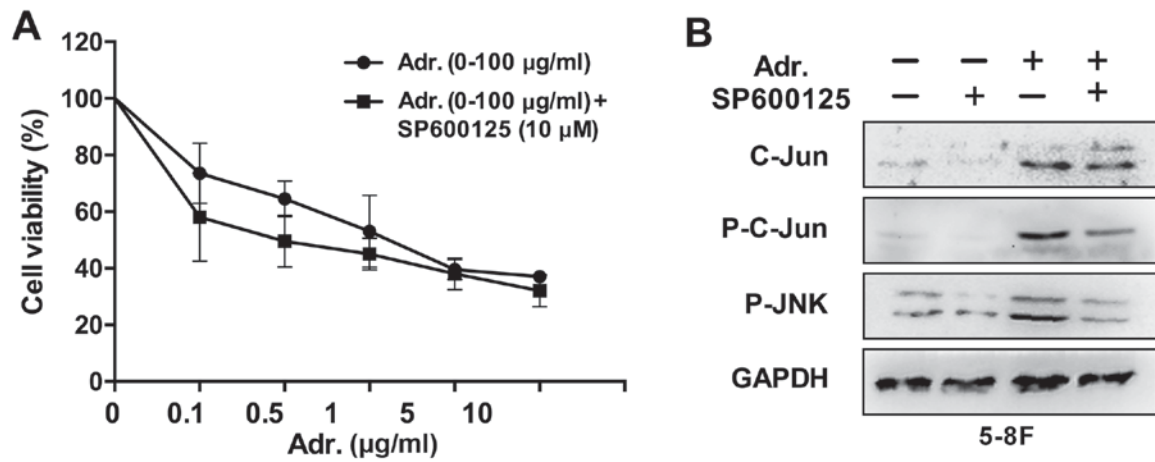


Figure 4. JNK pathway inhibition sensitized nasopharyngeal carcinoma cells to Adr. 5-8F cells were treated with 10  $\mu$ M SP600125 and increasing concentrations of Adr. (0-10  $\mu$ g/ml) for 24 h. (A) Cell viability was assessed by MTT assay. Data are expressed as the mean  $\pm$  standard deviation of at least triplicate experiments. (B) Western blot analysis demonstrated the activation of the JNK pathway. P-, phosphorylated; JNK, c-Jun N-terminal kinase; Adr., Adriamycin.

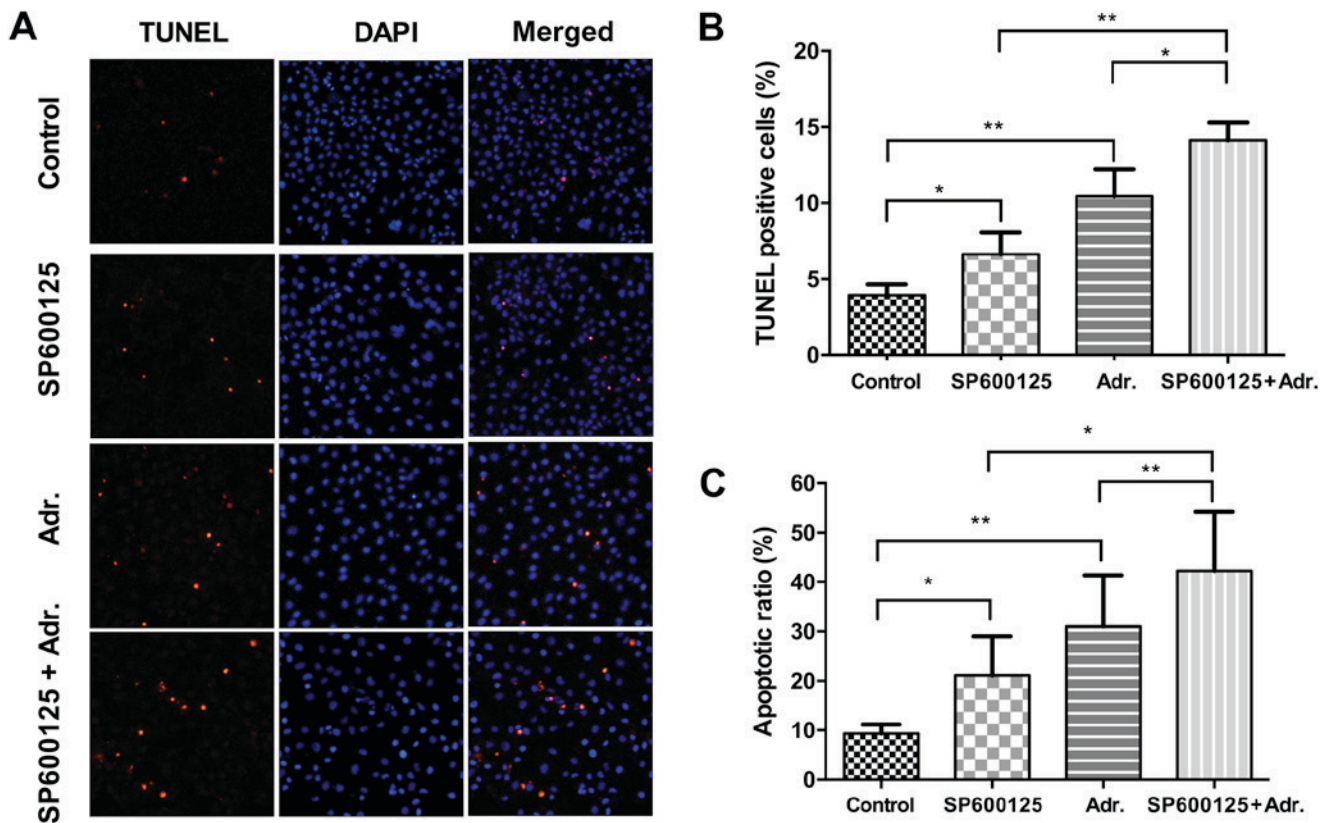


Figure 5. Adr. combined with the JNK pathway inhibitor induced apoptosis in nasopharyngeal cells. (A) TUNEL assay was used to examine the induced apoptosis at 24 h subsequent to treatment. Red dots represent TUNEL-positive cells, and blue dots represent nuclei staining by DAPI. The magnification was  $\times 100$ . (B) Apoptosis was expressed as the percentage of TUNEL-positive cells over the total number of cells. For quantification, a minimum of 3 slides and 3 fields on each slide were analyzed. (C) Quantification of apoptotic rate by flow cytometry. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  compared with the untreated group. P-, phosphorylated; JNK, c-Jun N-terminal kinase; Adr., Adriamycin.

rate, suggesting its pro-tumorigenic role and anti-apoptotic activity.

Overall, the present study showed that the inhibition of the JNK pathway sensitized NPC cells to the cytotoxic effects of Adriamycin, which resulted in cell growth inhibition and apoptosis. Since the function of the JNK pathway in cancer pathogenesis is dependent on cancer cell type, whether this

strategy may be applied to other cancers remains unknown, and additional studies are required.

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