

Effect of JIP on The Proliferation and Apoptosis of Nasopharyngeal Carcinoma Cells Through Interaction With JNK Mediated Pathway*

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Abstract Activator protein 1 (AP-1) is known to be constitutively activated by the Epstein-Barr latent membrane protein 1 in nasopharyngeal carcinoma cells. Increasing evidence indicated that C-jun N-terminal kinase (JNK), the key upstream kinase of AP-1 mediated signal transduction pathway, plays a key role in the carcinogenesis and progression of nasopharyngeal carcinoma. JNK interacting protein 1 (JIP-1) was newly identified as a potent inhibitor of JNK. The effect of JIP on the proliferation of nasopharyngeal carcinoma cells through interaction with the AP-1 signaling pathway was detected using immunofluorescence, reporter gene, MTT, colony formation and flow cytometric analysis. In nasopharyngeal carcinoma cells, data suggested that JIP down-regulated AP-1 activity through the inhibition of the translocation of phospho-JNK from the cytoplasm to the nucleus. Furthermore, JIP inhibited the rates of cell survival and colony formation. The number of cells in S phase decreased and the number of cells in G1/G0 phase increased after the flow cytometric analysis, suggesting that JIP induced growth arrest of Tet-on-LMP1-HNE2 cells in G1/S phase of the cell cycle. The results, therefore, demonstrated that JIP, by inhibiting AP-1-mediated signal transduction pathway, interfered the cell cycle and may act as an important negative regulator of the proliferation of nasopharyngeal carcinoma cells. Also, it was detected by flow cytometry analysis and laser scanning confocal microscope that JIP triggered the apoptosis of NPC cells. In conclusion, JIP represents a promising new therapeutic molecule for nasopharyngeal carcinoma.

Key words nasopharyngeal carcinoma, activator protein 1 (AP-1), c-jun N-terminal kinase, JNK interacting protein, proliferation, cell cycle, apoptosis

Nasopharyngeal carcinoma (NPC) is a kind of cell carcinoma common in South China, South-East Asia and North Africa. The etiological study of NPC has shown that Epstein-Barr virus (EBV) is closely associated with the carcinogenesis of nasopharyngeal epithelium^[1]. The latent membrane protein 1 (LMP1) encoded by EBV is involved in the transformation of B lymphocyte^[2]. The signaling pathway mediated by LMP1 played important roles in the cell proliferation, transformation and apoptosis^[3]. Recent studies showed that EBV encoded LMP1, which is similar to CD40^[4,5], was involved in the activation of C-jun N-terminal kinase (JNK) and its target molecule activator protein-1 (AP-1) which is key modulators of cell growth and also relevant to several tumors^[6].

AP-1 is a superfamily which contains c-Jun, JunB, c-fos, ATF2 and so on. The phosphorylation level of c-Jun controls the transcription and DNA binding of AP-1. As the key upstream kinase of AP-1,

JNK phosphorylates its subunits, c-Jun and JunB. Therefore JNK is the most important upstream regulator of AP-1 activity^[7]. Dickens^[8] found that JNK interacting protein 1 (JIP-1) was a potent cytoplasmic inhibitor of JNK. In addition, JIP-1 suppressed the JNK signaling pathway on cellular proliferation, including transformation by the Bcr-Abl oncogene. Previous studies have shown that in NPC cells LMP1 activates the JNK pathway through its C terminus via a mechanism involving TRADD and TRAF, and promotes the growth of NPC cells^[9,10]. Furthermore, in this study, we analyzed whether JIP could inhibit AP-1 signaling pathway

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mediated by JNK and what would be the effect of NPC cells treated by JIP.

1 Materials and Methods

1.1 Plasmids

The AP-1 luciferase reporter plasmid was a gift from Dr. LI (NCI, Frederick, USA). A sequence of the collagenase promoter region (−73 to +67) containing one AP-1 binding site was excised from the collagenase AP-1 CAT construct and inserted into a luciferase reporter construct pGL2-basic as reported^[1]. pcDNA3.1-Flag-JBD (JIP-1) was a gift from Dr. Miralles^[2].

1.2 Cell culture

The EBV negative low differentiated nasopharyngeal squamous carcinoma cell line HNE2^[3] and the dual-stable LMP1 integrated nasopharyngeal carcinoma cell line Tet-on-LMP1-HNE2^[4], in which the expression of LMP1 in EBV will be turned on by tetracycline and its derivatives such as Doxylane (Dox), were established by Cancer Research Institute, Xiangya School of Medicine. The cells were grown at 37°C in RPMI 1640 (GIBCO BRL), containing 15% fetal calf serum (FCS) and 100 U/ml penicillin/streptomycin. The Tet-on-LMP1-HNE2 cells were cultured in the above medium with 100 mg/L G418 and 50 mg/L hygromycin.

1.3 Transient transfection

1×10^5 of Tet-on LMP1 HNE2 cells in 2 ml of the appropriate complete growth medium were plated in each well of a 24-well plate (or 35mm dishes for immunofluorescence assay) for 24 h before transfection. When the cells are 50%~80% confluent, transfections were performed by lipofectamine according to the manufacturer's instruction (Gibco BRL). For each transfection, 2 μ g of AP-1 dependent luciferase reporter plasmid was diluted into 80 μ l serum-free media in a tube and 2 μ l lipofectamine into 120 μ l serum-free media in another tube. The two solutions were mixed gently and incubated at room temperature for 30~45 min. 200 μ l of the mixture and 150 μ l serum-free media were added into each well. The cells were incubated at 37°C for 4~5 h, and then the transfection media were replaced with fresh complete growth media.

1.4 Immunofluorescence assay

Tet-on LMP1 HNE2 cells with or without transfection were cultured in 35mm dishes for 24 h before the treatment with Dox. After the fixation for 15 min in 100% methanol at −20°C, the cells were washed with ice-cold PBS. The proportion of primary antibody to phospho-JNK (New England Biolabs) was 1:1 000 and detected with a secondary FITC-conjugated goat anti-rabbit antibody at 1:100.

1.5 AP-1 dependent reporter gene assay

AP-1 dependent reporter gene activity was detected by Luciferase Assay System (Promega, Inc.). Add 4 volumes of water to 1 volume of $5 \times$ lysis buffer. Carefully remove the growth medium from cells to be assayed. Rinse cells with cold $1 \times$ PBS buffer twice and remove as much of the PBS rinse as possible. Add 100 μ l of $1 \times$ Reporter Lysis Buffer per well to cover the cells. Rock culture dishes several times and scrape attached cells from the dish. The cell lysate was collected into a microcentrifuge tube on ice. After vortex the tube for 15 s and centrifuge at 12 000 g for 15 s at room temperature, the supernatant was transferred to a new tube. 20 μ l of cell extract was mixed with 100 μ l of Luciferase Assay Regent and the luciferase activity was measured for 20 s.

1.6 MTT Assay

To determine the effect of JIP on cell proliferation, HNE2-LMP1 cells were plated at a concentration of 1×10^5 cells in 48-well plates. At the indicated time after incubation, the MTT (1-[4, 5-dimethylthiazol-2-yl]-3, 5-dephenylformazan) assay was performed. 40 μ l of 5.0 g/L solution of MTT was added to each well. After incubation for 4 h at 37°C, the supernatant was removed and the reaction was terminated by 200 μ l DMSO, after which the absorbance at 570 nm was measured.

1.7 Colony formation assay

500 indicated cells were seeded each well of 6-well plates and allowed to grow for 14 days. Remove the growth medium from cells and wash cells with $1 \times$ PBS buffer twice. The colonies were fixed with 100% methanol for 15 min and stained with 0.4% crystal violet. Colonies of ≥ 50 cells were counted under microscope. The plating efficiency was determined by the formulation: plating efficiency (PE)=(the number of colonies/the total seeded cells) $\times 100\%$.

1.8 Cell cycle analysis

Cell cycle phase distribution was analyzed by cytometry using PI staining. Briefly, control for treated cells was harvested by trypsinization, washed with $1 \times \text{PBS}$, then fixed in 70% ethanol, and then treated with 50 mg/L RNase A for 30 min at 4°C . Resulting DNA distributions were analyzed on a FACSsort (Becton Dickinson, San Jose, CA) with Cell-Quest software (version 3.3) for the proportions of cells in G0-G1, S phase and G2-M phases of the cell cycle.

1.9 Apoptosis analysis with flow cytometry and confocal

Apoptosis was determined by sub-G1 content, as indicated by flow cytometry. Briefly, adherent cells were harvested by trypsinization, counted, and washed with PBS. Cells were then fixed in the cold 70% ethanol at a concentration of $1 \times 10^6/\text{ml}$ with samples not exceeding 3×10^6 cells. Cells were fixed for at least 1 h before adding of 0.5 ml of 18 mg/L PI containing 40 mg/L RNase A.

2 Results

2.1 Retention of phospho-JNK in the cytoplasm of NPC cells with JIP

To demonstrate the mechanism involved in JNK interacting with JIP, we investigated the subcellular

localization of phospho-JNK immunofluorescence assay in the NPC cells. An antibody specific to the phospho-JNK (Thr183/Tyr185) detected high immunoactivity of JNK in the nucleus of Tet-on-LMP1-IINE2 cells induced by Dox (Figure. 1b) and relative low immunoactivity without Dox (Figure. 1a). After treatment with JIP, the phospho-JNK was detected mainly in the cytoplasmic of NPC cells (Figure. 1c). These showed that the phospho-JNK aggregated mainly in the nucleus and JIP caused the retention of phospho-JNK in the cytoplasmic of NPC cells.

2.2 Down-regulation of JIP to AP-1 activity

JIP caused cytoplasmic retention of phospho-JNK that may lead to the inhibition of JNK-regulated gene expression and activity. To determine whether the AP-1 activity was changed after JIP introduction, we approached it through transient transfection with AP-1 luciferase reporter gene and different quantities of JIP plasmid (0.5~4 μg). Really high AP-1 activity was detected in Tet-on-LMP1-IINE2 cells. After treatment with JIP, the transactivity of AP-1 triggered by LMP1 was significantly down-regulated by JIP in a dose-dependent manner. This data showed that JIP could obviously down-regulate the AP-1 activity induced by LMP1 in NPC cells (Figure. 2).

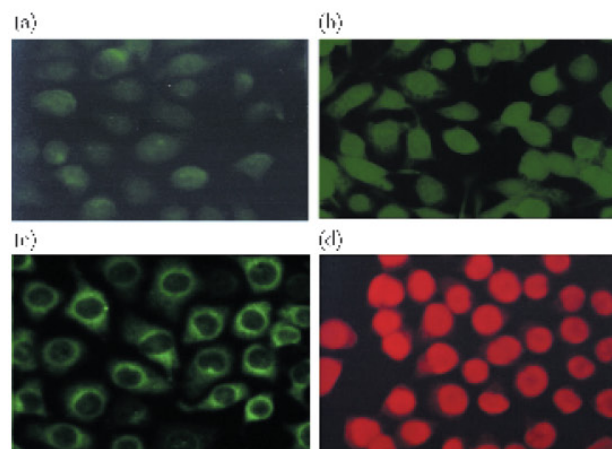


Fig. 1 JIP inhibited the translocation of phospho-JNK

After Tet on LMP1 IINE2 transfection with JIP for 24 h, subcellular location of phospho JNK was determined by immunofluorescence analysis of cell culture with JNK (Thr183/Tyr185) antibody and a FITC conjugated goat anti rabbit second antibody. (a) Tet on LMP1 IINE2 cells not induced by Dox; (b) Tet on LMP1 IINE2 cells induced by Dox; (c) Tet on LMP1 IINE2 cells transfected with JIP plasmid; (d) Tet on LMP1 IINE2 cells counterstained with PI.

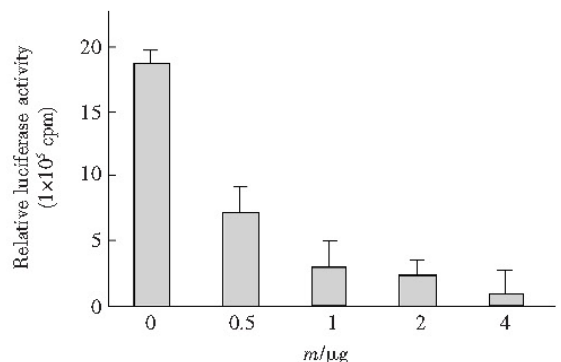


Fig. 2 Down-regulation of the activity of the AP-1 by different quantities of JIP expression plasmid in nasopharyngeal carcinoma cells

Tet on LMP1 IINE2 cell were cotransfected with 2 μg of AP 1 luc and different dose of JIP. The graphs represent the means and standard deviations of three parallel wells from three individual experiments.

2.3 Inhibition of the proliferation of NPC cells by JIP

To further determine the effect of JIP on the

proliferation of Tet-on LMP1 IINE2 cells, we used the MTT assay and colony formation assay. Data showed that survival cells were significantly inhibited in a dose - and time-dependent manner after transfection with JIP (Figure. 3). The rate of colony formation decreased sharply 14 days after the transfection of the JIP expression plasmid into Tet-on-LMP1 IINE2 cells (Figure. 4).

2.4 Induction G1/S arrest of NPC cells by JIP

We applied the flow cytometry analysis to demonstrate the mechanism involved in the inhibition of the proliferation of NPC cells by JIP. Flow cytometric analysis of the cells for DNA content showed that compared with untreated cells, the number of cells in S phase decreased and the number of cells in G1/G0 phase increased, suggesting that JIP induced cell cycle arrest of Tet-on-LMP1 IINE₂ cells in G1/S boundary (Figure. 5).

2.5 Enhancement of the apoptosis of NPC cells by JIP

Our study showed that JIP arrest the G1/S phase of the cell cycle may contribute to one of the mechanisms of the inhibition of NPC cells. Furthermore, we detected the apoptosis of nasopharyngeal carcinoma cells after transient transfection with JIP plasmid by flow cytometry and laser confocal microscope. Data showed that the apoptosis rates of NPC cells increased from 1.25% to 8.25% at 24 h and from 1.04% to 31.45% at 48 h after transfection with JIP plasmid (Figure. 6). The confocal assay showed that the morphology of nucleus changed in NPC cells (Figure. 7). All suggested that JIP triggered the apoptosis of NPC cells.

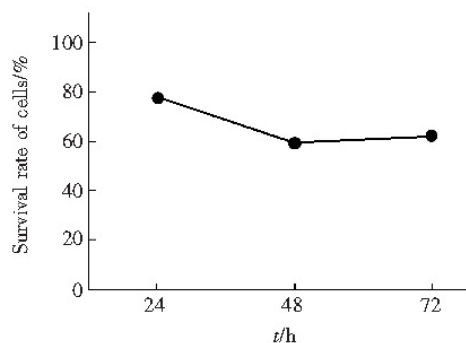


Fig. 3 Effect of JIP on survival rate of Tet-on LMP1 IINE2 cells at different time

Tet on LMP1 IINE2 cells were plated at 1×10^5 cells each well in the 48 well plate, and MTT assay was performed on day 1, 2, 3 after induction with Dox. The survival rate of cells decreased in the 48 h after transfection with JIP.

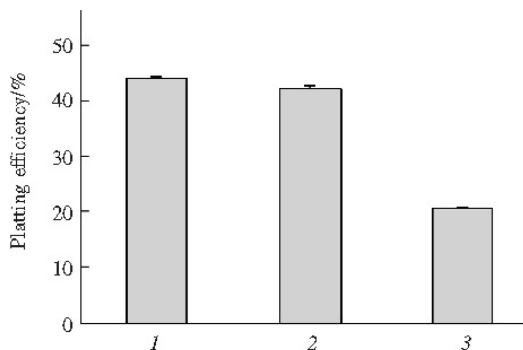
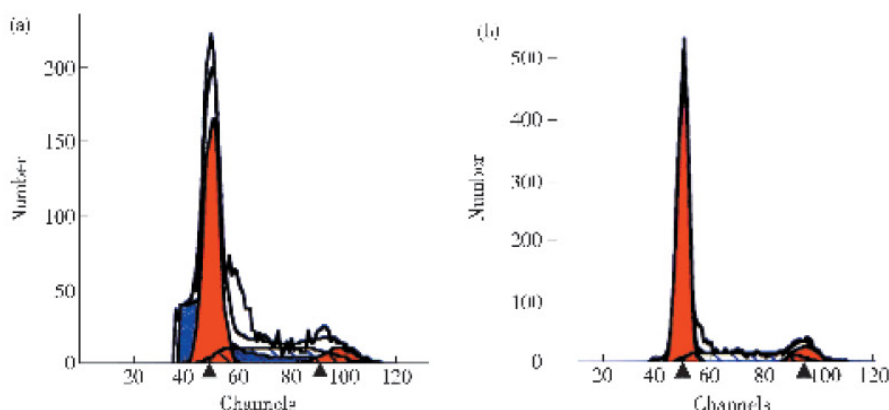


Fig. 4 Colony formation assay of Tet-on LMP1 IINE2 cells

Cells were seeded in 6 well plates at 500 cells each well and allowed to grow for 14 days. The colonies were fixed with 100% methanol and stained with 0.4% crystal violet. Colonies of ≥ 50 cells were counted. 1: Untransfection; 2: Control (transfection with ssDNA); 3: Transfection with JIP.



FFig. 5 Cell cycle distribution of Tet-on LMP1 IINE2 cells induced by Dox after treatment with JIP

After transfection with JIP an additional 2 days, the cells were fixed with 70% ethanol, stained with propidium iodide and analyzed by flow cytometry for DNA content. The fraction of cells with G1/S and G2/M DNA content are shown. (a) Tet on LMP1 IINE2 cells induced with Dox; (b) Tet on LMP1 IINE2 cells transfected by JIP with induction of Dox.

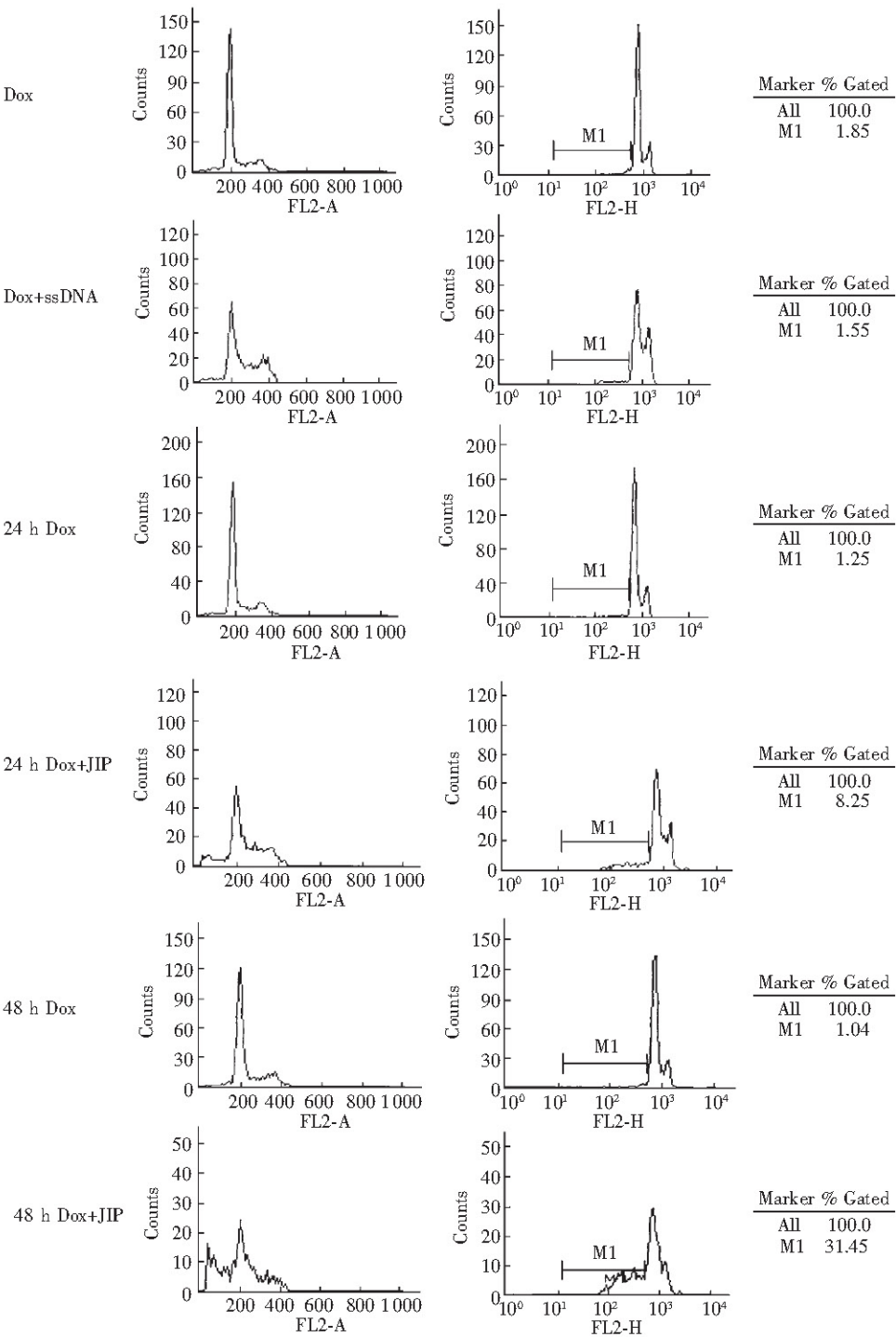


Fig. 6 Apoptosis rate of Tet-on LMP1 HNE2 cells at different time after treatment with JIP

After transfection with JIP at 24 h in Tet-on LMP1 HNE2 cells, the ratio of apoptosis increased from 1.25% to 8.25% as compared with the cells treatment with Dox. Also the apoptosis ratio increased from 1.04% to 31.45% at 48 h after treatment with JIP.



Fig. 7 Variation of nucleus of nasopharyngeal carcinoma cells observed with laser scanning confocal microscope
(a) Tet-on-LMP1 HNE2 cells induced by Dox; (b) Tet-on-LMP1 HNE2 cells transfected by JIP with induction of Dox; (c) control (transfected with ssDNA).

3 Discussion

The cytoplasmic protein JIP 1 binds selectively to the MAP kinase JNK but not to other MAPK. Overexpression of JIP 1 causes cytoplasmic retention of JNK and thereby inhibits gene expression mediated by the JNK signaling pathway [15]. Our previous study has shown that LMP1 activates AP 1 mediated by JNK signaling pathway in NPC cells. Results suggested that JIP down regulated the activity of AP 1 through inhibition of the translocation of phospho JNK from the cytoplasm to the nucleus, which implied that the inhibition of JNK pathway by expressing JIP may cause the biological effect of NPC cells. We detected the effect of JIP on the proliferation of nasopharyngeal carcinoma cells using MTT, colony formation, and flow cytometric analysis. MTT assay showed that cell proliferation was significantly inhibited in a dose and time dependent manner. The rate of colony formation was decreased 14 days after the transfection of JIP expression plasmid into Tet on LMP1 HNE₂ cells. Our results showed that the number of cells in S phase was decreased and the number of cells in G1/G0 phase increased as determined by the flow cytometric analysis, suggesting that JIP induced growth arrest of Tet on LMP1 HNE₂ cells in G1/S phase of the cell cycle. Therefore, data demonstrated that JIP, by inhibiting AP 1 mediated signaling transduction pathway, interfered the cell cycle and may act as an important negative regulator of proliferation of nasopharyngeal carcinoma cells.

Tumor cells may activate survival pathways that function dominantly with respect to the JNK pro apoptotic pathway. Alternatively, the targets of pro apoptotic JNK pathway may be altered to

suppress JNK dependent apoptosis. Finally, it is possible that, in the context of tumor cell, JNK may be interpreted as an anti apoptotic or growth signal [16]. Bost *et al* [17] found that antisense of JNK oligonucleotides inhibited the growth of tumor cells and may induce apoptosis.

In summary, our study indicated that in nasopharyngeal carcinoma cells, JIP could completely inhibit the JNK signal pathway activated by EBV LMP1. The expression of JIP could inhibit the *in vitro* growth and the proliferation of nasopharyngeal carcinoma cells, which might be involved in the G1/S phase cell cycle arrest and the process of apoptosis. These studies imply that JIP represents a new promising therapeutic target for nasopharyngeal carcinoma.

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JNK 相互作用蛋白通过 JNK 途径 影响鼻咽癌的增殖和凋亡*

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摘要 EB病毒编码的瘤蛋白潜伏膜蛋白(LMP1)所介导的活化蛋白(AP-1)信号转导途径在细胞增殖、分化、转化与凋亡方面发挥着重要作用。越来越多的证据表明, AP-1信号转导通路中上游激酶JNK在鼻咽癌的发生发展过程中起着重要作用。最近克隆出来的JNK相互作用蛋白(JIP-1)是一种能抑制JNK核移位的胞浆锚蛋白。为探讨JIP在LMP1调控AP-1信号通路中的作用机制, 采用间接免疫荧光法和报告基因法, 发现JIP通过有效地抑制磷酸化的JNK从胞浆移位入核, 从而抑制LMP1上调的AP-1活性。同时, JIP导入鼻咽癌细胞中, MTT法发现JIP能够明显抑制鼻咽癌细胞的生长。进一步发现转染JIP后细胞的集落形成率与对照组相比大约降低了53.6%, 也抑制了细胞。提示JIP可明显抑制细胞的增殖作用。进一步采用流式细胞术分析, 结果发现JIP引起细胞G1/S期细胞阻滞, 说明JIP是抑制细胞增殖的重要调节子。进一步采用流式细胞术定量发现, 转染JIP后细胞的24 h凋亡百分率由1.25%上升到8.25%, 上升约6.6倍, 48 h由1.04%上升到31.45%, 上升约30倍。采用激光共聚焦显微镜发现, 转染JIP后细胞核发生显著变化, 核质由均匀状态固缩成高凝集状态, 形成了典型的胞膜体。提示JIP可有效地促进细胞凋亡。结果表明, JIP可通过抑制活化的JNK核移位, 降低LMP1所介导的AP-1信号通路。并进一步发现JIP可有效地抑制细胞增殖和细胞凋亡, 从而提示JIP可作为新的治疗肿瘤潜在靶分子。

关键词 鼻咽癌, 活化蛋白1, JNK, JNK相互作用蛋白, 增殖, 细胞周期, 凋亡

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