

Original Article

Effects of ERK/MAPK signal transduction pathway on proliferation and apoptosis of nasopharyngeal carcinoma

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Abstract: Nasopharyngeal carcinoma is one Epstein-Barr virus (EB)-related malignant tumor. Molecular study showed critical roles of extracellular signal-regulated kinase/mitogen-activated protein kinases (ERK/MAPK) signal transduction pathway in proliferation, differentiation, and apoptosis inhibitor of malignant tumor cells. As basic biological phenomena of cells, apoptosis is under regulation of various cytokines. This study recruited nasopharyngeal carcinoma patients and CNE-2Z cells to detect expression of proliferation, apoptosis related proteins, in order to analyze the effect of ERK/MAPK signal transduction pathway on proliferation and apoptosis of nasopharyngeal cells and related mechanisms. Nasopharyngeal carcinoma patients in our hospital were recruited. Expression of phosphorylated ERK (p-ERK) and Ki-67 proteins was examined from nasal mucosa tissues. Nasopharyngeal CNE-2Z cells were treated by PD98059, followed by MTT, TUNEL and western blot assays for cell proliferation, apoptosis and expression of p-ERK and Ki-67. Patient group had 80% positive rate of p-ERK and 70% positive rate of Ki-67 ($p < 0.05$ compared to adjacent or control tissues). PD98059 treated cells had lower proliferation activity, higher apoptosis, decreased pERK1/2 and Ki-67 expression ($p < 0.05$ compared to control group). In nasopharyngeal patients, p-ERK1/2 and Ki-67 were up-regulated. PD98059 inhibited proliferation of CNE-2Z cells, facilitated cell apoptosis, and inhibited ERK1/2 phosphorylation. In conclusion, ERK signaling pathway participates in the proliferation and apoptosis of nasopharyngeal carcinoma.

Keywords: ERK/MAPK, nasopharyngeal carcinoma, proliferation, apoptosis

Introduction

Nasopharyngeal carcinoma is mainly occurred in Southern China, and is one epithelial derived malignant tumor in head-neck region. Its occurrence is mainly related with Epstein-Barr virus (EB) infection, and is also predisposed to distal metastasis [1]. During occurrence and development of malignant tumors, cell apoptosis is one important and basic biological activity. Its occurrence is under co-regulation of multiple genes and cytokines, including commonly occurred Bcl-2 family, Caspase family, oncogene and tumor suppressor gene. During growth, differentiation and death process, and cell necrosis or tumor formation, they all exert critical roles with close correlation [2]. During biological process of malignant transformation, activation of oncogenes is one important inducing factor, whilst multiple signal transduc-

tion pathways are important regulatory steps. Mitogen-activated protein kinase (MAPK) mainly exists in eukaryotes, and can be activated by multiple cytokines, growth factors, and activated neurotransmitter, further initiating expression levels of transcriptional factors, protein kinase and regulatory genes, thus modulating cell proliferation, migration and apoptosis with rapidness and sequence [3, 4]. In a study about signal transduction pathway, MAPK signal transduction pathway consists of four sub-families, including extracellular signal regulatory kinase (ERK), c-Jun amino terminal kinase (JNK/SAPK), p38 MAPK and ERK5/BMK1 [5]. In various human malignant tumors such as lung cancer and breast cancer, activated ERK exists and locates in chromosome 1p34-35. Its phosphorylated forms, p-ERK, has elevated expression in all these malignant tumor cells [6]. Ki-67 is one common marker for cell prolif-

eration. During different stages of cell cycles, its expression level is different. Usually, Ki-67 starts to show positive expression at late stage of G1 phase, and gradually elevates till S phase, eventually reaching the peak at M phase. With normal mitosis, Ki-67 protein is rapidly degraded and lower expression [7]. In various studies about nasopharyngeal carcinoma, it is still unclear whether ERK or Ki67 has effects on proliferation and apoptosis of nasopharyngeal. This study thus recruited nasopharyngeal carcinoma patients as the research object, plus CNE-2Z cells as experimental model to test cell proliferation, apoptosis and p-ERK protein expression, in order to analyze the effect and mechanism of ERK/MAPK signal transduction pathway on proliferation and apoptosis of nasopharyngeal carcinoma cells.

Materials and methods

General information

A total of 40 nasopharyngeal carcinoma patients in our hospital from January 2015 to January 2016 were recruited as the research subjects. All patients were diagnosed as nasopharyngeal carcinoma, including 20 males and 20 females, aging between 25 and 75 years (average age of 46.1 ± 9.4 years). Another cohort of 40 patients without nasopharyngeal carcinoma were recruited as the control group, including 12 males and 8 females, aging between 20 and 70 years (average age of 45.1 ± 3.4 years). No significant difference in sex or age existed between two groups of patients ($p > 0.05$), which were thus comparable.

Inclusive criteria: All patients were diagnosed by pathology examination. Patients had no mesenchymal disease or immune disorder, have not received radio- or chemo-therapy, cell-immunity therapy, frozen or laser treatment. Patients had no disease in major organs such as heart, liver and lungs, or any acute/chronic infectious diseases.

The study protocol was approved by the Research Ethics Committee of Second Clinical Medical College of Fujian and all patients gave their informed consent before study commencement.

Experimental cells

Nasopharyngeal carcinoma CNE-2Z cell line was purchased from Immunology Department, Fourth Military Medicine University.

Reagent and equipment

p-ERK1/2 and Ki-67 primary antibody kit and DAB test kit were purchased from Maixin Biotech (Shanghai, China). P98059 (Promega, USA). Rabbit anti-mouse p-ERK1/2 and Ki-67 polyclonal antibody, goat anti-rabbit secondary antibody and TUNEL assay kit were purchased from Santa Cruz Biotech. (Santa Cruz, CA, USA). DMEM medium, streptomycin/penicillin and fetal bovine serum were purchased from Gibco BRL. Co. Ltd. (Grand Island, New York, USA). Incubator (Thermo Electron Corp, Waltham, MA, USA). CO₂ incubator (SANYO, Japan). Inverted microscope (Nikon, Japan). Cold high-speed centrifuge (Beckman, USA). -80°C fridge (SANYO, Japan).

Immunohistochemistry for detecting p-ERK1/2 and Ki-67 expression in patient nasal mucosal tissues

Tissues were fixed, immersed in paraffin and embedded. Tissue slices were prepared, dewaxed and rehydrated. After heat antigen retrieval, tissues were blocked and incubated in primary antibody (1:200) for 1 h, followed by secondary antibody (1:100) for 10 min incubation. DAB substrate was added to develop the slice. After quenching, counterstaining was performed, followed by differentiation, mounting and capturing images.

p-ERK1/2 and Ki-67 positive staining was defined as no staining in nucleus, and brown to yellow brown granules in cytoplasm or membrane. Based on positive stained cell number, negative (-) was defined as less than 10% of positive cells; Weak positive (+) was defined as 11%~25% of stained cells; Positive (++) was defined as 26%~50% of positive cells; Strong positive (+++) was defined as >50% of positive cells.

Normal cell culture

Nasopharyngeal carcinoma CNE-2Z cells were cultured in 1640 medium containing 10% FBS in a 37°C chamber with 5% CO₂ with fixed humidity. Cells were digested in 0.25% trypsin and were seeded into 24-well plate with 200 µL per well containing 10⁵ cells/mL. When cells reached 80% confluence, cells were continuously cultured for 24 h using 1% FBS. Culture medium was changed based on cell growth status for passage.

PD98059 treatment on nasopharyngeal CNE-2Z cells

Nasopharyngeal carcinoma CNE-2Z cells at log-growth phase were counted and inoculated into culture plate. Under normal incubation, cells were attached and incubated overnight, followed by 2% FBS incubation for 24 h. DMEM medium containing 10% FBS was changed. Experimental group received 50 $\mu\text{mol/L}$ PD98059 for 1 h treatment. Control group used untreated CNE-2Z cells.

MTT assay for nasopharyngeal carcinoma CNE-2Z cells proliferation activity

Nasopharyngeal carcinoma CNE-2Z cells at log-growth phase were adjusted and seeded into culture plate at 8×10^4 per well. Cells were cultured in a 37°C chamber with 5% CO_2 . After 12 h, 24 h and 48 h, cell activity was measured, followed by MTT incubation (20 μL at 5 mg/mL) for 4 h. After stopping the incubation, 150 μL DMSO was added for 10 min vortex. Absorbance (A) values at 570 nm were measured in a microplate reader. Using A values as vertical axis, and time as horizontal axis, a growth curve was plotted.

TUNEL assay for apoptosis of CNE-2Z cells

Tissues were dehydrated and embedded in paraffin, followed by sectioning. TUNEL method was used to label apoptotic nucleus. In brief, tissues were immersed in xylene, and washed in gradient in ethanol. Proteinase K working solution was used to incubate tissues. 50 μL TUNEL reaction mixture was added. The number of apoptotic cells was counted. 50 μL converter-POD was added, followed by 50~100 μL DAN substrate and counter staining by hematoxylin or methyl-green. Cells were counted and imaged. Brown granules in the nucleus were identified as positive marker. Five high-magnification fields were randomly selected and were observed under light field microscope. Number of positive cells and total number of cells were counted for calculating apoptotic rate (AR).

Western blot for p-ERK1/2 and Ki-67 protein expression in CNE-2Z cells

After drug treatment, culture medium was discarded and was washed by cold PBS for three times. Pre-cold RIPA lysis buffer (containing 50

mmol/L Tris-HCl at pH 7.4, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, sodium orthovanadate, NaF, EDTA and leupeptin). 0.1 mg/mL PMSF was added before use, for lysing cells using aprotinin and phosphotransferase by 10 min iced incubation. Proteins were scratched by spoons and were lysed in ultrasound. The mixture was centrifuged at 4°C for 12000 r/min for 30 min. The supernatant was saved and stored at -80°C for further use. Protein samples were extracted and mixed with loading buffer, and were boiled at 100°C for 5 min. Proteins were separated by electrophoresis using 5% stacking gel and 10% SDS-PAGE separation gel, and were transferred to PVDF membrane using 10 V semi-dry method. The membrane was washed in TBST (50 mmol/L Tris-HCl at pH 7.6, 150 mmol/L NaCl and 0.1% Tween 20) for 5 min, and was blocked at 4°C overnight. Primary antibody (β -actin, ERK1/2 and pERK1/2) was added for 37°C incubation for 2 h, followed by four times of PBST washing. Horseradish peroxidase (HRP) conjugated goat anti-rabbit or goat anti-mouse secondary antibody was added for 1 h incubation at room temperature. After TBST washing for three times, substrate was added for development, followed by exposure and imaging in Alphamager TM2200. Density value was measured and was presented as the ratio of gray values against β -actin.

Data processing

SPSS17.0 statistical software was used for data processing. Measurement data was analyzed by chi-square test. Measurement data was presented as mean \pm standard variation and processed in analysis of variance (ANOVA). A statistical significance was defined when $p < 0.05$.

Results

Immunohistochemistry for p-ERK and Ki-67 expression in patient nasal mucosal tissues

p-ERK1/2 and Ki-67 expression were measured in all patients. In experimental group, 18 patients had strong positive expression of p-ERK and 14 of them had positive expression with overall positive rate at 80%. Ki-67 expression assay showed 12 strong positive and 17 positive cases, reaching 70% positive rate, which was higher than adjacent tissues and control group ($p < 0.05$, **Table 1, Figure 1**).

Signal molecules in tumor apoptosis

Table 1. p-ERK1/2 and Ki-67 expression in patient tissues (n, %)

Group	n	p-ERK1/2 expression strength				Ki67 expression strength			
		-	+++	+++	Positive rate (%)	-	+++	+++	Positive rate (%)
Cancer	40	8	14	18	80*,#	12	17	11	70*,#
Adjacent	40	32	8	0	20	17	3	0	15
Control	40	36	4	0	10	36	4	0	10

Note: *, p<0.05 compared to tumor adjacent tissues; #, p<0.05 compared to control group.

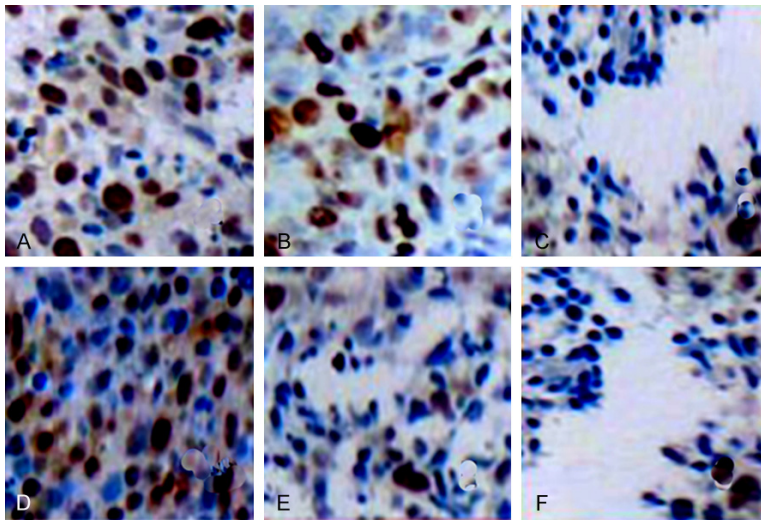


Figure 1. Expression of p-ERK1/2 and Ki-67 in patient tissues ($\times 400$). A. p-ERK1/2 expression in tumor tissues (+++); B. p-ERK1/2 expression in tumor adjacent tissues (+); C. p-ERK1/2 in control group (-); D. Ki-67 expression in cancer tissues (+++); E. Ki-67 expression in tumor adjacent tissues (+); F. Ki-67 expression in control group.

Table 2. MTT assay for CNE-2Z cell proliferation activity

Time	PD98059 group	Untreated group
6 h	0.143 \pm 0.016*	0.201 \pm 0.013
12 h	0.102 \pm 0.011*,#	0.202 \pm 0.012
24 h	0.086 \pm 0.024*,#,&	0.234 \pm 0.011

Note: *, p<0.05 compared to untreated group; #, p<0.05 compared to 6 h group; &, p<0.05 compared to 12 h group.

MTT assay for CNE-2Z cell proliferation activity

Proliferation activity of nasopharyngeal carcinoma CNE-2Z cells was measured. Compared to control group, proliferation activity of PD98059 group was gradually decreased (p<0.05, Table 2).

TUNEL assay for apoptosis of nasopharyngeal carcinoma CNE-2Z cells

24 h after intervention, apoptosis of nasopharyngeal carcinoma CNE-2Z cells was measured.

Compared to untreated group, PD98059 cells had elevated cell apoptotic rate (p<0.05, Table 3, Figure 2).

Expression of p-ERK1/2 and Ki-67 proteins in nasopharyngeal carcinoma CNE-2Z cells

Expression of p-ERK1/2 and Ki-67 proteins in pharyngeal carcinoma CNE-2Z cells was measured. Compared to untreated group, PD98059 group had lower expression of p-ERK1/2 and Ki-67 proteins (p<0.05, Table 4, Figures 3, 4).

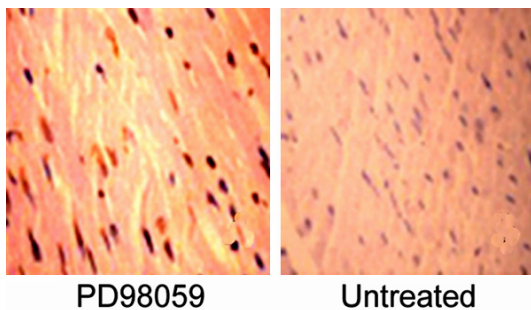
Discussion

Basic study found the involvement of MAPK signal transduction pathway in various patho-physiological process of the body. It also participates in regulation of cell proliferation, differentiation and apoptosis, and is the convergence of multiple pathways. Its activation is under regulation of various mitogens including growth factors and transcriptional factors. When MAPK signal transduction pathway is activated, it rapidly translocate into the nucleus for further response to facilitate cell proliferation and growth [8]. Among four members of MAPK family, ERK signal pathway has drawn research interests. Once being activated, ERK signal transduction pathway includes kinase such as GTPase, Ras, Raf-1 and MEK1/2, which can translocate signals to the nucleus from membrane via receptors, further regulating cell proliferation and apoptosis [9]. ERK1/2 mainly locates in the cytoplasm. After being activated by mitogen, ERK1/2 can penetrate the nuclear membrane for phosphorylation in the nucleus. Phosphorylated ERK1/2 functions on respective transcriptional factors to regulate gene transcrip-

Table 3. TUNEL assay for CNE-2Z cell apoptosis

Time	PD98059 group	Untreated group
6 h	10.27±1.44*	3.01±1.26
12 h	19.48±1.67*,#	3.21±1.43
24 h	21.85±2.12*,#,&	3.32±1.39

Note: *, p<0.05 compared to untreated group; #, p<0.05 compared to 6 h group; &, p<0.05 compared to 12 h group.

**Figure 2.** TUNEL assay for cell apoptosis.

tion, thus mediating protein expression level, and exerting critical functions for normal cellular metabolism and functions [10, 11]. This study recruited nasopharyngeal carcinoma patients in our hospital as the research objects, along with nasopharyngeal carcinoma cell line CNE-2Z, to analyze the effect of ERK/MAPK signal transduction pathway for cell proliferation and apoptosis along with related mechanism.

In this study, nasal mucosa tissues were collected from nasopharyngeal carcinoma patients and normal people for measuring p-ERK1/2 and Ki-67 expression in tissues. We found higher positive rate of p-ERK1/2 and Ki-67 positive rate in nasopharyngeal carcinoma patients' tumor tissues than those in adjacent or control tissues. These results indicated up-regulation of p-ERK1/2 and Ki-67 in nasopharyngeal carcinoma patients. When ERK signal transduction pathway in nasopharyngeal carcinoma patients is continuously activated, ERK can modulate occurrence and progression of nasopharyngeal carcinoma.

This study cultured nasopharyngeal carcinoma CNE-2Z cells, which were treated by PD98059. Cell proliferation and apoptosis were analyzed. Compared to untreated group, PD98059 group had lower proliferation activity and higher apoptosis, indicated that PD98059 could suppress proliferation and growth of nasopharyn-

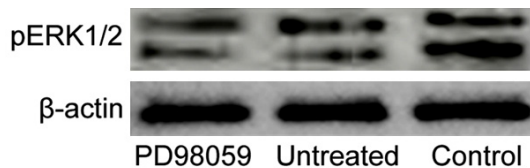
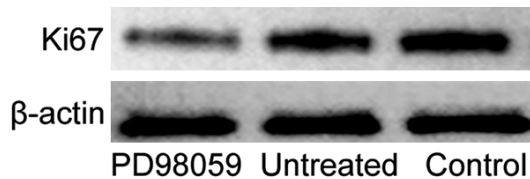
geal carcinoma CNE-2Z cells, thus facilitating cell apoptosis. Cell mitogen including growth factors, cytokines, and transcriptional factors can activate ERK. Such process might be achieved by Ras-Raf-MEK system. Phosphorylated ERK1/2 can activate other protein kinase in the cytoplasm, facilitating phosphorylation of cytoskeleton components [12]. ERK signal antagonist PD98059 can suppress ERK1/2 activation to certain extents, as it can bind with ATP binding sites of MEK1, thus exerting specific blocking effects without affecting total ERK expression level. *In vitro* study showed that PD98059 could significantly suppress cancer cell growth [13]. ERK/MAPK signal transduction pathway is believed to be one key pathway for cell motility and proteinase secretion, whilst PD98059 could partially impede ERK/MAPK signal transduction pathway, thus exerting certain effects on biological processes such as cell proliferation, differentiation, apoptosis, infiltration and invasion [14].

Extracellular signal regulatory protein kinase (ERKs) is co-induced by multiple cytokines and growth factors. ERK1/2 is the most critical factor in regulating cell proliferation, differentiation and cell cycle [15]. Previous study indicated that once body presented constitutive activation of ERK1/2, cell proliferation may be enhanced. This is mainly dependent on ERK1/2 activation status. The constitutive activation of ERK1/2 has synergistic effects with other signal transduction pathway, participating in cell elements and downstream molecules, to facilitate cell survival or to induce cell death [16-18]. Previous study showed as on marker for cell proliferation, Ki-67 can indicate proliferation of nasopharyngeal carcinoma cells, and predict the prognosis of patients [19]. This study tested p-ERK1/2 and Ki-67 protein expression in CNE-2Z cells. Compared to untreated group, PD98059 group had decreased expression of p-ERK1/2 and Ki-67 proteins, indicating that PD98059 can exert certain inhibitory effects on protein expression level and ERK1/2 phosphorylation. Previous study showed that activated ERK/MAPK signal transduction pathway can phosphorylate RSK proteinase, trans-activate cAMP response binding protein, co-agonist CBP, c-fos, serum reaction factor and ER, all of which facilitate cell proliferation [20]. PD98059 can partially arrest activation level of p-ERK1/2, but does not affect total ERK protein

Table 4. Protein expression of p-ERK1/2 and Ki-67 in CNE-2Z cells

Group	pERK1/2	Ki67
PD98059 group	0.078±0.002*,#	0.113±0.013*,#
Untreated group	0.103±0.004	0.228±0.015
Control group	0.102±0.004	0.304±0.015

Note: *, p<0.05 compared to untreated group; #, p<0.05 compared to control group.

**Figure 3.** p-ERK1/2 protein expression in CNE-2Z cells.**Figure 4.** Ki-67 protein expression in CNE-2Z cells.

expression levels. Therefore, phosphorylated activation of ERK/MAPK and up-regulation of signal transduction related protein expression are both caused by proliferation, growth and infiltration of malignant tumors [21], as consistent with our results.

Conclusion

p-ERK1/2 and Ki-67 are highly expressed in nasopharyngeal carcinoma patients. PD98059, as one antagonist of ERK/MAPK signal transduction pathway, it can inhibit proliferation of nasopharyngeal carcinoma CNE-2Z cells to facilitate cell apoptosis, thus inhibiting related protein expression level and ERK1/2 phosphorylation to certain extents. Occurrence of nasopharyngeal carcinoma involves the interconnection between multiple cytokines, cellular factors and signal transduction pathway, especially involving multiple disease occurrence and progression, and the interaction with target genes, consisting a complex mechanism. Detailed mechanisms of ERK/MAPK in occurrence and progression of nasopharyngeal carcinoma still requires further illustration, thus providing evidences for clinical promotion.

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Disclosure of conflict of interest

None.

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