

## Original Article

# The PK3K/AKT and ERK/MAPK signaling pathways correlate with nasopharyngeal carcinoma

Rong Yang<sup>1</sup>, Hai Ge<sup>1</sup>, Fengxian Liu<sup>1</sup>, Hong Jiang<sup>2</sup>, Jing Liu<sup>1</sup>

Departments of <sup>1</sup>Otolaryngology, <sup>2</sup>General Surgery, Qingdao Third People's Hospital, Qingdao 266000, Shandong, China

Received August 16, 2018; Accepted January 12, 2019; Epub July 15, 2019; Published July 30, 2019

**Abstract:** Nasopharyngeal carcinoma is a common tumor in southern China. Its occurrence/progression is correlated with EB viral infection. A molecular study showed the participation of the PI3K/AKT and ERK/MAPK signal transduction pathways in the proliferation and apoptosis of malignant tumors. This study thus selected nasopharyngeal patients and cultured CNE-2Z model cells to test cell apoptosis and the expressions of related proteins. The implications of the PI3K/AKT and ERK/MAPK signal pathways in nasopharyngeal carcinoma is also illustrated. Nasopharyngeal carcinoma patients were recruited to measure their AKT, ERK1/2, p-AKT, and p-ERK1/2 expressions. CNE-2Z cells were treated with Wortmannin and/or PD98059. Cell morphology, apoptosis, and the expressions of AKT, ERK1/2, p-AKT, and p-ERK1/2 proteins were measured by fluorescence, TUNEL assay and Western blot, respectively. Nasopharyngeal carcinoma tissues had a 70% positive rate of AKT, a 60% rate of ERK1/2, 80% of p-AKT, and 70% p-ERK1/2 positive expression ( $P < 0.05$  compared to the control or adjacent tissues). The CNE-2Z cells in the experimental group had an elevated apoptosis rate and a higher expression of AKT, ERK1/2, p-AKT, and p-ERK1/2 ( $P < 0.05$  compared to control group). PD98059 and the Wortmannin group had enhanced CNE-2Z cell apoptosis plus higher AKT and ERK1/2 expressions and lower p-AKT or p-ERK1/2 expression compared to either PD98059 or the Wortmannin group. All three groups showed potentiated apoptosis over time. p-AKT and p-ERK1/2 are up-regulated in nasopharyngeal carcinoma tissues. The down-regulation of PK3K/AKT and ERK/MAPK facilitated CNE-2Z cell apoptosis.

**Keywords:** PK3K/AKT, ERK/MAPK, nasopharyngeal carcinoma, apoptosis

## Introduction

Nasopharyngeal carcinoma frequently occurs in southern China and is a common cancer in head-neck malignant tumors. Without timely diagnosis and treatment, it frequently leads to distal metastasis, thus severely threatening patients' health. However, due to the complex anatomical structure of the nasopharyngeal cavity, carcinoma usually has insidious symptoms, which are frequently ignored. Many patients are already at the terminal stage when they are diagnosed, at which time about 5%~10% patients already have distal metastasis. Among stage IV patients, about 40% of the cases are at stage Iva, and more than 50% are at stage IVb [1, 2]. Focal invasion and distal metastasis are important biological features of malignant tumors, involving multiple genes and cytokines. A previous study showed the participation of the PK3K/AKT and ERK/MAPK signal transduction pathways in the onset and pro-

gression of multiple malignant tumors, and these pathways have become a research focus for tumor treatment. The PK3K/AKT signal transduction pathway has been found to participate in the anti-apoptotic process of malignant tumor cells. Meanwhile, it can also regulate the normal progression of cell cycles, activate telomerase, and regulate angiogenesis, thus participating in cell invasion/migration, and playing a critical role in the proliferation, differentiation and apoptosis of malignant tumor cells [3, 4]. Extracellular signal regulatory kinase (ERK) is one important member of the MAPK family. A previous study showed the participation of the ERK1/2 signal transduction pathway in the proliferation of malignant tumors. In ovary cancer, gastric carcinoma and the pulmonary cancer ERK1/2 is highly expressed. Its phosphorylation and consequent activation can penetrate across nuclear membranes, enhancing the expression of down-

## Molecular markers of nasopharyngeal cancer

stream signal molecules such as cyclin D1 and Ki-67, thus facilitating the over-proliferation of malignant tumor cells and transformation. Studies in breast cancer patients found that the up-regulation of p-ERK1/2 phosphorylated activity in cultured breast gland epithelial cells could facilitate the transformation of normal epithelial cells into malignant tumor cells [5-7], indicating that the participation of abnormal activation of ERK1/2 in the occurrence and progression of malignant tumors. Both of those have been found to be expressed in nasopharyngeal carcinoma cells, leaving its detailed mechanisms unclear. This study recruited nasopharyngeal carcinoma patients and CNE-2Z cells for analyzing the effect of the PK3K/AKT and ERK/MAPK signal transduction pathways in the onset and progression of nasopharyngeal carcinoma.

### Materials and methods

#### General information

A total of 40 nasopharyngeal carcinoma patients who were admitted to Qingdao Third People's Hospital (Qingdao, Shandong, China) from January 2017 to January 2018 were recruited as the experimental group, which included 20 males and 20 females, between 25 and 75 years old (average age =46.1±9.4 years). All patients were diagnosed by pathology, including 10, 18, and 12 cases of phase I, phase II phase III patients. Another cohort of 20 patients with benign nasopharyngeal lesions in the General Hospital of Daqing Oil Field during the same period was included in the control group, which consisted of 12 males and 8 females (average age =45.1±3.4 years). No significant difference existed in sex or age between two groups, which were thus comparable ( $P > 0.05$ ).

The study protocol was approved by the Research Ethics Committee of Qingdao Third People's Hospital (Qingdao, Shandong, China), and all patients gave their informed consent before the study began.

#### Experimental cells

The nasopharyngeal carcinoma cell line CNE-2Z was obtained from the Heilongjiang University of Chinese Medicine.

#### Experimental reagents

AKT, ERK1/2, p-AKT, a p-ERK1/2 primary antibody kit, and a DAB test kit were purchased

from Maixin Biotech (China). Wortmannin and P98059 were purchased from Promega (US). Rabbit anti-mouse AKT, ERK2/2, p-AKT, and p-ERK1/2 polyclonal antibody, and goat anti-rabbit secondary antibody were purchased from Gibco (US).

#### IHC staining for expression of AKT, ERK1/2, p-AKT and p-ERK1/2 in nasal mucosal tissues

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

AKT, ERK1/2, p-AKT and p-ERK1/2 positive staining was defined as no staining in the nuclei, and brown to yellow brown granules in the cytoplasm or membranes. Based on the 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; and strong positive (+++) was defined as > 50% of positive cells.

#### Experimental grouping

PD98059 group: Nasopharyngeal carcinoma CNE-2Z cells were cultured in a 1640 medium containing 10% FBS in a 37°C chamber with 5% CO<sub>2</sub> at fixed humidity. After digestion in 0.25% trypsin, the cells were inoculated in a 24-well plate at 200 µL per well containing 10<sup>5</sup> cells. When the cells reached 80% confluence, they were cultured in 1% FBS medium for 24 h for passage. CNE-2Z cells at the log-growth phase were counted and inoculated into culture plates for attached growth overnight. 2% FBS was added for a 24 h culture, followed by incubation in a DMEM medium with 10% FBS. PD98059 (50 µmol/L) was added for 1 h treatment, followed by continuous incubation.

Wortmannin group: The cells were cultured as described above. Wortmannin (100 µmol/L) was added for 1 h intervention, followed by continuous incubation.

PD98059 + Wortmannin group: The cells were cultured as described above. 50 µmol/L PD98059 and 100 µmol/L Wortmannin were

## Molecular markers of nasopharyngeal cancer

**Table 1.** The expression of AKT, ERK1/2, p-AKT, and p-ERK1/2 in nasal mucosal tissues by IHC

Item	Experiment group (n=40)		Control group (n=40)
	Cancer tissue	Tumor adjacent tissue	
AKT			
-	12	17	38
+-++	17	3	2
+++	11	0	0
Positive rate (%)	70 <sup>a,b</sup>	15	5
p-AKT			
-	8	32	36
+-++	14	8	4
+++	18	0	0
Positive rate (%)	80 <sup>a,b</sup>	20	10
ERK1/2			
-	16	36	38
+-++	16	4	2
+++	18	0	0
Positive rate (%)	60 <sup>a,b</sup>	10	5
p-ERK1/2			
-	12	17	36
+-++	17	3	4
+++	11	0	0
Positive rate (%)	70 <sup>a,b</sup>	15	10

Note: a, P<0.05 compared to adjacent tissues; b, P<0.05 compared to the control group.

added for 1 h intervention, followed by continuous incubation.

Untreated group: Naïve nasopharyngeal carcinoma CNE-2Z cells were cultured as described as above.

Control: Nasal mucosal epithelial cells were collected from patients without nasopharyngeal carcinoma and were cultured as described above.

### Dual fluorescence staining method for CNE-2Z cells

The cells were cultured using a routine method and were intervened as described above. Hoechst 33342/PI dual fluorescent labeling was performed for observing the cell apoptosis.

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

The tissues were dehydrated and embedded in paraffin, followed by sectioning. The TUNEL

method was used to label the apoptotic nuclei. In brief, tissues were immersed in xylene, and washed in gradient in ethanol followed by HCl immersing. Proteinase K working solution was used to incubate the tissues. The TUNEL reaction mixture was added, and it was incubated for 1 h under 37°C with coverslips. A converter-POD was added for 30 min incubation at 37°C with coverslips. An NBT/NCIP substrate was added for 30 min of dark incubation. The reactions were quenched and developed, dehydrated and mounted. The apoptotic cell nuclei were stained dark blue. A colored pathology imaging system was used for the analysis.

### Western blot for AKT, ERK1/2, p-AKT, and p-ERK1/2 protein expression in CNE-2Z cells

The cells were washed and lysed in 50 µL RPIA lysis buffer at 0°C for 10 min of iced incubation. The proteins were scratched, lysed and centrifuged to collect the supernatant, which was stored at -80°C. Proteins were mixed with a loading buffer and were boiled at 100°C for 5 min. The proteins were separated by electrophoresis and transferred to a PVDF membrane, where they were blocked at 4°C overnight. A primary antibody (β-actin, p-AKT and p-ERK1/2 at 1:100 dilution) was added for 2 h incubation. The membrane was washed four times, followed by incubation in a goat anti-rabbit secondary antibody for 1 h. The membrane was then washed three times, developed and exposed for image analysis.

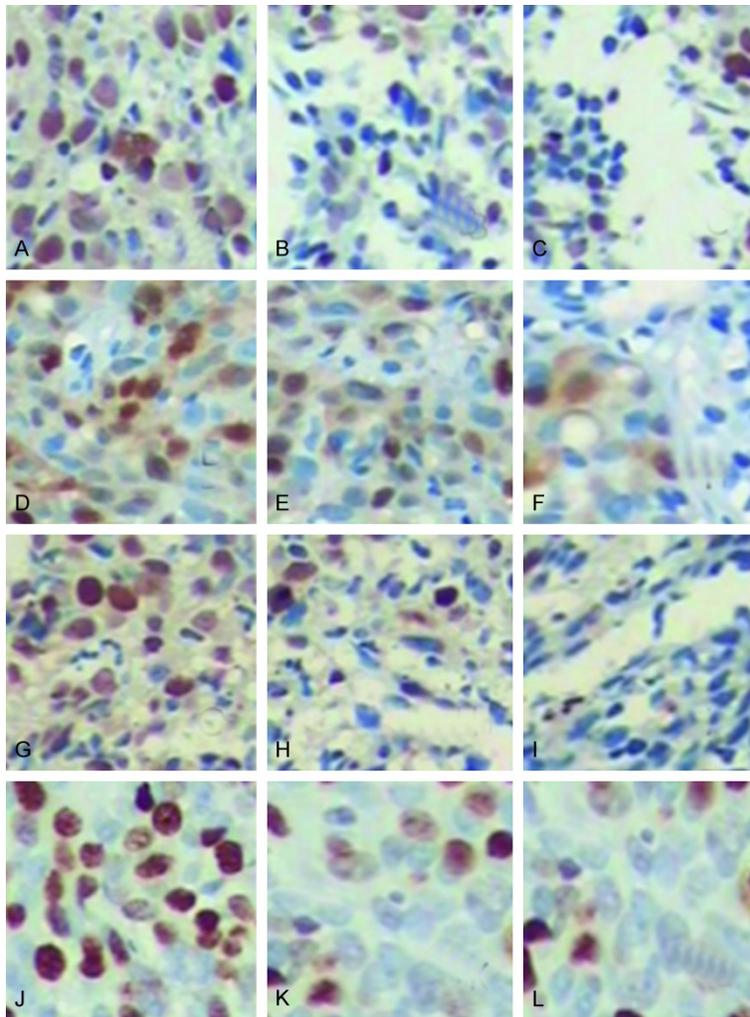
### Data processing

SPSS 17.0 statistical software was used for data processing. The enumeration data were tested using a chi-square analysis. The measurement data were processed using an analysis of variance (ANOVA) and were presented as the mean ± standard deviation (SD). Statistical significance was defined when P<0.05.

## Results

### IHC staining for AKT, ERK1/2, p-AKT and p-ERK1/2 expressions in nasal mucosal tissues

The expressions of AKT, ERK1/2, p-AKT, and p-ERK1/2 in patient tissues were measured. The experimental group had a 70% positive rate of AKT expression, 60% of ERK1/2 positive expression, 80% of p-AKT positive expression, and 70% of p-ERK1/2 expression, all of which were higher than the adjacent tissues or the control group (P<0.05, **Table 1; Figure 1**).



**Figure 1.** The expressions of AKT, ERK1/2, p-AKT, and p-ERK1/2 in patient tissues ( $\times 400$ ). A. AKT expression in tumor tissues (+++); B. AKT expression in adjacent tissues (+); C. AKT expression in the control group (-); D. ERK1/2 expression in the cancer tissues (+++); E. ERK1/2 expression in the adjacent tissues (+); F. ERK1/2 in the control group (-); G. p-AKT expression in cancer tissues (+++); H. p-AKT expression in adjacent tissues (+); I. p-AKT expression in the control group (-); J. p-ERK1/2 expression in tumor tissues (+++); K. p-ERK1/2 expression in adjacent tissues (+); L. p-ERK1/2 expression in the control group (-).

*Dual fluorescent staining for the CNE-2Z cells after 24 h intervention*

24 h after intervention, the CNE-2Z cells in the PD98059 group and the Wortmannin group showed swelling and smeared boundaries. In the PD98059 + Wortmannin group, the CNE-2Z cells showed condensation of the cytoplasm, shrinkage and rounding of the cells, with a worse reflective property, and partial ruptures of the cell membranes. With an elongated intervention time, the cells showing abnormal morphology increased (**Figure 2**).

*TUNEL assay for CNE-2Z cell apoptosis*

The apoptosis of the nasopharyngeal carcinoma CNE-2Z cells was measured in all the groups. The experimental group had higher apoptosis than the control group ( $P < 0.05$ ). The PD98059 + Wortmannin groups had elevated CNE-2Z cell apoptosis than PD98059 or Wortmannin group ( $P < 0.05$ ). Comparing between 6 h, 12 h, and 24 h showed gradually increased apoptosis as time elapsed ( $P < 0.05$ , **Table 2**).

*Western blot for AKT, ERK1/2, p-AKT and p-ERK1/2 protein expression in CNE-2Z cells*

We measured the protein expression of AKT, ERK1/2, p-AKT, and p-ERK1/2 in the CNE-2Z cells after 24 h intervention. The experimental CNE-2Z cells had higher protein expressions of AKT, ERK1/2, p-AKT, and p-ERK1/2 ( $P < 0.05$  compared to control group). The PD + Wortmannin group had higher expressions of AKT and ERK1/2 proteins, plus lower p-AKT or p-ERK1/2 protein than PD98059 or the Wortmannin group ( $P < 0.05$ , **Table 3**; **Figure 3**).

**Discussion**

Nasopharyngeal carcinoma frequently occurs in Southern China. Derived from nasal mucosal epithelial cells, nasopharyngeal carcinoma is mainly treated by radiation therapy. Although it has a relatively higher focal management rate, nasopharyngeal carcinoma frequently has focal recurrence or distal metastasis. The control of the focal recurrent rate and the improvement of the long-term survival rate have become a major challenge for research [8]. Molecular studies have showed the participation of the PI3K/AKT signal transduction pathway in the regulation of the malignant tumor cell cycle, affecting telomerase activity, angiogenesis,

## Molecular markers of nasopharyngeal cancer

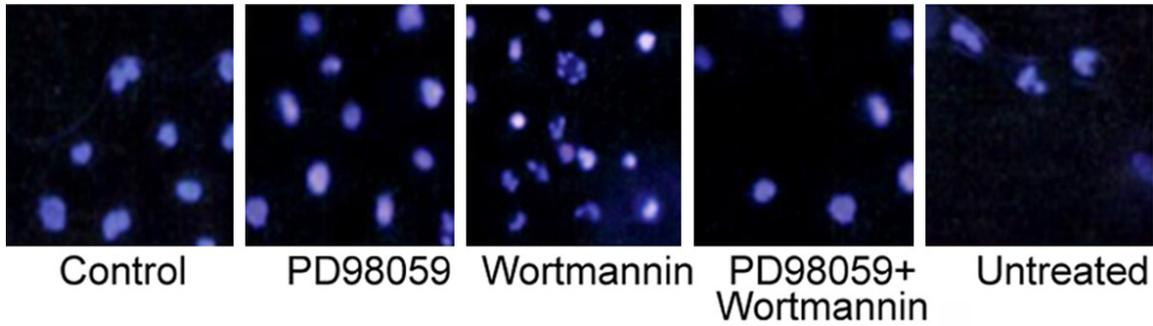


Figure 2. CNE-2Z cell morphology after 24 h intervention.

Table 2. Apoptosis of CNE-2Z cells in all groups

Group	6 h	12 h	24 h
Experiment			
PD98059	5.63±0.25 <sup>a,b</sup>	6.89±0.35 <sup>a,b,e</sup>	8.03±0.67 <sup>a,b,e,f</sup>
Wortmannin	8.51±0.47 <sup>a,b,c</sup>	9.62±1.36 <sup>a,b,c,e</sup>	11.04±1.19 <sup>a,b,c,e,f</sup>
PD98059 + Wortmannin	15.87±2.07 <sup>a,b,c,d</sup>	19.89±2.24 <sup>a,b,c,d,e</sup>	23.64±2.47 <sup>a,b,c,d,e,f</sup>
Untreated	3.13±0.21	3.20±0.18	3.17±0.21
Control	1.54±1.11	1.59±1.09	1.76±1.10

Note: a, P<0.05 compared to untreated group; b, P<0.05 compared to control group; c, P<0.05 compared to experimental group A; d, P<0.05 compared to experimental group B; e, P<0.05 compared to 6 h; f, P<0.05 compared to 12 h.

and invasion/migration, all of which are important biological processes [9, 10]. Once being phosphorylated, active ERK1/2 can activate the transcriptional factor, regulating the protein transcription process and gene expression, mediating divisions and the proliferation or apoptosis of malignant tumor cells [11-13]. This study thus recruited nasopharyngeal carcinoma patients and CNE-2Z cells, which were intervened by the ERK/MAPK inhibitor PD98059, and/or the PK3K/AKT inhibitor Wortmannin for intervention, to analyze the effect of the PK3K/AKT, ERK/MAPK signal transduction pathway in the onset and prgression of nasopharyngeal carcinoma and related mechanisms.

In this study, nasopharyngeal carcinoma patients in our hospital were recruited as the experimental group, in parallel with a control group without nasopharyngeal carcinoma. The experimental group had a 70% positive expression of AKT expression, a 60% positive expression of ERK1/2, an 80% positive expression of p-AKT and a 70% positive expression of p-ERK1/2, all of which were elevated compared to the tumor tissues or the adjacent tissues. These results indicated an over-expression of p-AKT and p-ERK1/2 in nasopharyngeal carcinoma, and their participation in tumor onset and occurrence.

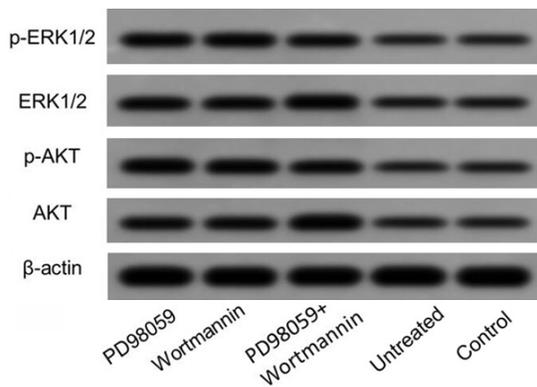
This study further cultured nasopharyngeal carcinoma CNE-2Z cells, which were intervened by the ERK/MAPK inhibitor PD98059 and/or the PK3K/AKT inhibitor Wortmannin. The Dual fluorescent staining method observed the unclear boundary of CNE-2Z cells in the PD98059 and Wortmannin group. The PD98059 + Wortmannin group had condensed cytoplasm, shrinkage of cells, rupture, or membranes and more cells with abnormal morphology as time elapsed. Further tests examined the apoptosis of CNE-2Z cells. The experimental group had elevated apoptosis compared to the control group. The PD98059 + Wortmannin group had higher CNE-2Z cell apoptosis than the PD98059 or Wortmannin group. Comparing among 6 h, 12 h, and 24 h showed the gradually increased apoptosis of CNE-2Z cells in all three subgroups. The ERK signal transduction pathway was shown to be up-regulated in various malignant tumors including pulmonary cancer, prostate carcinoma, and melanoma [14, 15]. The PI3K/AKT signal transduction pathway has also been shown to be up-regulated in malignant tumors. Once activated, it can phosphorylate related substrates, among which PI3K has been confirmed to participate in cell proliferation, differentiation, and apoptosis. AKT is under the regulation of PI3K. Its phosphorylation at

## Molecular markers of nasopharyngeal cancer

**Table 3.** Expression of AKT, ERK1/2, p-AKT and p-ERK1/2 proteins in CNE-2Z cells

Group	AKT	ERK1/2	pAKT	p-ERK1/2
Experiment				
PD98059	0.373±0.004 <sup>a,b</sup>	0.389±0.004 <sup>a,b</sup>	0.372±0.002 <sup>a,b</sup>	0.383±0.005 <sup>a,b</sup>
Wortmannin	0.294±0.003 <sup>a,b,c</sup>	0.401±0.006 <sup>a,b,c</sup>	0.315±0.004 <sup>a,b,c</sup>	0.404±0.003 <sup>a,b,c</sup>
PD98059 + Wortmannin	0.525±0.002 <sup>a,b,c,d</sup>	0.578±0.003 <sup>a,b,c,d</sup>	0.228±0.002 <sup>a,b,c,d</sup>	0.213±0.003 <sup>a,b,c,d</sup>
Untreated	0.122±0.002	0.137±0.002	0.175±0.001	0.128±0.001
Control	0.115±0.001	0.113±0.001	0.112±0.001	0.106±0.001

Note: a, P<0.05 compared to the untreated group; b, P<0.05 compared to the control group; c, P<0.05 compared to the PD98059 group; d, P<0.05 compared to the Wortmannin group.



**Figure 3.** AKT, p-AKT, ERK1/2 and p-ERK1/2 protein expression in CNE-2Z cells after treatment.

Thr308 and Ser473 sites and further abnormal activation can facilitate the proliferation of malignant tumor cells as it inhibits their apoptosis [16, 17]. The results of this study showed that the inhibition of the PK3K/AKT and ERK/MAPK signal transduction pathway could facilitate the apoptosis of nasopharyngeal carcinoma CNE-2Z cells. Jiang et al. found that the application of LY294002 to inhibit the PI3K/Akt signal pathway could arrest the cell cycle G1 phase and eventually facilitate cell apoptosis [18], a finding consistent with this study.

This study tested the protein expressions of AKT and p-ERK1/2 in nasopharyngeal carcinoma CNE-2Z cells. The experimental group had a higher expression of AKT, ERK1/2, p-AKT and p-ERK1/2 than the control group. PD98059 + the Wortmannin group had a higher protein expression of AKT and ERK1/2 than PD98059 or the Wortmannin group, while the p-AKT and p-ERK1/2 protein expressions were lower. This study showed that the inhibition of the PK3K/AKT and ERK/MAPK signal transduction pathways could decrease p-AKT or p-ERK1/2 expression while inhibiting ERK1/2 phosphorylation in CNE-2Z cells. Some studies have shown

that the continuous activation of ERK1/2 might lead to increased cell proliferation, mainly depending on the ERK1/2 activation status, which may have synergistic effects with other signal transduction pathways, related cell elements and downstream molecules to facilitate cell survival or induce cell death [19, 20]. An *In vitro* study showed that PD98059 could inhibit the growth of malignant tumor cells. This might be due to the binding between PD98059 and the MEK1 ATP site, thus impeding the phosphorylation of ERK. As one specific inhibitor of AKT, Wortmannin can inhibit AKT phosphorylation to suppress the *in vitro* growth of cell lines [21], which is consistent with our results.

### Conclusion

p-AKT and p-ERK1/2 are highly expressed in nasopharyngeal carcinoma tissues. Inhibition of the PK3K/AKT and ERK/MAPK signal transduction pathways could facilitate the apoptosis of CNE-2Z cells, decrease p-AKT or p-ERK1/2 expression, and suppress ERK1/2 phosphorylation to a certain extent. The PK3K/AKT and ERK/MAPK signal transduction pathways exert important roles in the onset and progression of nasopharyngeal carcinoma. Detailed mechanisms targeting the roles of the PK3K/AKT and ERK/MAPK signal transduction pathways in the occurrence of nasopharyngeal carcinoma still requires further investigation to provide evidence for clinical treatment.

### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Rong Yang, Department of Otolaryngology, Qingdao Third People's Hospital, No. 29, Yongping Road, Licang District, Qingdao 266000, Shandong, China. Tel: +86-0532-84612090; Fax: +86-0532-84612090; E-mail: zankdu31282@126.com

## Molecular markers of nasopharyngeal cancer

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