# CDK3 expression and its clinical significance in human nasopharyngeal carcinoma

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Abstract. The aim of the current study was to investigate the expression of cyclin-dependent kinase 3 (CDK3) in human nasopharyngeal carcinoma (NPC) and to evaluate its association with the clinicopathological characteristics of patients with NPC. CDK3 expression was examined in three NPC cell lines and one nasopharyngeal epithelial cell line by western blot analysis and in 94 specimens of NPC and 40 specimens of inflamed nasopharyngeal tissue by immunohistochemistry staining. CDK3 was overexpressed in the three NPC cell lines, 5-8F, CNE1 and CNE2, compared with the NP-69 nasopharyngeal epithelial cell line, and was primarily expressed in the cytoplasm. The frequency of CDK3 expression was significantly higher in NPC specimens (67%) compared with inflamed nasopharyngeal tissue specimens (12.5%; P<0.001). CDK3 expression was associated with the degree of infiltration, lymph node metastasis and tumor node metastasis clinical staging, respectively, (P<0.001) in patients with NPC. These results revealed that the expression of CDK3 is associated with the progression of NPC, and may be a potential biomarker for prediction of the prognosis of patients with NPC.

## Introduction

Nasopharyngeal carcinoma (NPC) is a cancer of head and neck squamous cells and commonly observed in Southeast Asia, including certain regions of South China, such as Guangdong, Guangxi, Hunan and Fujian provinces (1,2). According to the International Agency for Research on Cancer, there were an estimated 84,000 incident cases of NPC and

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51,600 mortalities due to NPC in 2008, with a reported annual incidence of  $30-80/10^5$  individuals in endemic regions (3). At present, the main treatment strategy for NPC is radiotherapy; however, patients with advanced disease tend to experience therapeutic failure and the 5 year overall survival rate is ~52% (4). Since NPC exhibits highly aggressive behavior (5) with rapid progression to mortality (6), it is essential to gain an improved understanding of the molecular events underlying the development of these tumors further improve survival rates. Thus, it further investigations into the molecular mechanisms of NPC are required.

The development and progression of NPC involves uncontrolled and indefinite proliferation, which is partially caused by abnormal cell cycle regulation. The change in cell cycle distribution, as well as abnormal expression and activity of associated regulatory factors, including cell cycle protein (cyclin), cyclin-dependent kinase (CDK) and its inhibitor cyclin-dependent kinase inhibitor (CKI), may result in cell cycle disorder and abnormal cell proliferation. CDK3, a member of the CDK family, was originally classified as a CDK due to its high sequence identity (76%) with CDC2 and CDK2 (7). CDK3 is critical in cell cycle regulation and is involved in G0-G1 and G1-S stage cell cycle transitions (8-13). Furthermore, CDK3 was overexpressed in a number of cancer cell lines, and may be important role in cell proliferation and malignant transformation (14-17).

CDK3 has been previously reported to be highly expressed in human glioblastoma (15). Ectopic expression of CDK3 enhances the transformation of JB6 Cl41 cells, and knockdown of endogenous CDK3 suppressed the proliferation and growth of T98G glioblastoma cells in soft agar (15), indicating that the CDK3 is important in tumorigenesis. However, the expression of CDK3 in NPC is unclear. The aim of the present study was to determine the association between CDK3 expression and the clinicopathological features of patients with NPC.

## Materials and methods

*Cell lines and tissue samples.* CNE1, CNE2 and 5-8F NPC cell lines and the NP-69 nasopharyngeal epithelial cell line were provided by Professor Tie-Bang Kang (Sun Yat-Sen University Cancer Center, Guangzhou, China) and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10%

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fetal bovine serum (both from Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C in a 5% CO<sub>2</sub> incubator. A total of 134 specimens were provided by the Department of Otolaryngology, Beijing University Shenzhen Hospital (2010-2012), with signed consent forms. Among these, there were 94 cases of NPC (61 males and 33 females). The age of patients ranged between 18-78 years (median age, 44 years). There were a total of 40 cases of nasopharyngeal inflammation (20 males and 20 females), the age of patients ranged between 23-68 years (median age, 42.6 years). All specimens were examined by clinical and pathological diagnosis.

All specimens were fixed with 10% formalin, embedded in paraffin within three days and processed into  $4-\mu m$ sections. Rabbit polyclonal antibodies against CDK3 were purchased from Abcam (Cambridge, MA, USA). An Ultra Sensitive <sup>TM</sup>S-P Allergic kit (mouse/rabbit) and a DAB Color Development kit were purchased from Fuzhou Manxin Biological Technology Development, Inc. A Leica Application Suite Microscope (Leica Microsystems GmbH, Wetzlar, Germany) was used to capture images of the stained sections.

Western blotting. Cells were harvested at 80-90% confluency, washed twice with cold phosphate-buffered saline (PBS) and lysed on ice in NP-40 cell lysis buffer (50 mmol/l Tris-HCl, pH 8.0, 150 mmol/l NaCl and 0.5% NP-40) with soybean trypsin inhibitor (Sigma, St. Louis, MO, USA). Protein concentration was measured using the detergent-compatible protein assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Equal quantities of protein were separated electrophoretically on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membrane was hybridized with the anti-CDK3 antibody (Abcam), and visualized using an enhanced chemiluminescence detection kit (Amersham Biosciences, Piscataway, NJ, USA). The membranes were stripped and reprobed with an anti-actin mouse monoclonal antibody (1:2,000 dilution; Millipore) as a loading control.

*Immunohistochemistry (IHC)*. Hematoxylin and eosin staining was performed on tissue samples to identify histopathological properties and pathological classifications. Paraffin sections were dewaxed and hydrated, the nucleus was stained with hematoxylin and the cytoplasm was stained with 0.5% eosin. Following dehydration, sections were sealed with transparent and neutral gum. The staining results were carefully examined by experienced pathologists to determine the tumor node metastasis staging according to the Chinese clinical staging scheme of NPC in 2008 (18).

IHC was used to detect the expression of CDK3 in tissue samples. Paraffin sections were dewaxed and hydrated; sections were submerged in 0.01 mmol/l citrate buffer and microwaved for antigenic retrieval for 20 min. Next, the antigens were cooled naturally, specimens were washed with PBS and incubated for 10 min at room temperature with endogenous peroxidase blockers (3% hydrogen peroxide in methanol; Professional-Bio, Shenzhen, China). Following washing with PBS, normal non-immune animal serum (goat) was added and specimens were incubated for 10 min at room temperature. Specimens were washed with PBS and incubated for 60 min at



Figure 1. Expression of CDK3 was elevated in NPC cell lines. Expression analysis of CDK3 protein in the NP-69 nasopharyngeal epithelial cell line and NPC cell lines (5-8F, CNE1 and CNE2) by western blotting. Protein expression levels were normalized with  $\beta$ -actin. CDK3, cyclin-dependent kinase 3; NPC, nasopharyngeal carcinoma.

room temperature with 3  $\mu$ g/ml primary antibody. Specimens were washed with PBS and incubated for 10 min at room temperature with the secondary, biotin-labeled antibody (goat anti-mouse IgG antibody; Abcam). Following washing with PBS, streptavidin-biotin immunoperoxidase solution (Abcam) was added and the specimens were incubated for 10 min at room temperature. Following washing with PBS, a color test was performed using the DAB Color Development kit (Zhongshan Golden Bridge, Beijing, China). The nucleus was stained with hematoxylin. Following dehydration, sections were sealed with transparent and neutral gum. PBS was used as a negative control instead of a primary or secondary antibody. The results were observed by microscopy. The cytoplasm or nuclei that were attached to brown or yellow particles were defined as positive cells. Ten representative high-power fields, selected randomly, were counted and each field counted was no less than 100 cells. Scores were determined by combining the proportion of positively stained carcinoma cells and the intensity of staining. The proportion of positively stained carcinoma cells was respectively scored as 1, <25% positive carcinoma cells; 2, 25-50% positive carcinoma cells; and 3, >50% positive carcinoma cells. The cells at each staining intensity were recorded on a scale of 0, no staining; 1, weak staining, light yellow; 2, moderate staining, yellowish brown; and 3, marked staining, brown. The staining index was calculated as follows: staining index = staining intensity x proportion of positively stained carcinoma cells. Scores were attributed symbols as follows: 0, (-); 1-2, (+); 3-4, (++); and 6-9, (+++). Negative cells were defined as (-) and positive cells were defined as (+), (++) or (+++).

Statistical analysis. Statistical analysis was performed using SPSS, version 15.0 software (SPPS Inc., Chicago, IL, USA). The difference between two groups was compared with a t-test. The  $\chi^2$  test was used to measure the significance of the association between the NPC specimens and nasopharyngeal inflammation specimens. A four-fold table correlation was analyzed with Pearson's correlation analysis. The correlation of CDK3 expression with the clinical pathologic parameters of the patients was analyzed using the Wilcoxon test or Spearman's correlation test.

## Results

*CDK3 expression in NPC and nasopharyngeal epithelial cell lines.* The expression pattern of CDK3 in cultured human NPC cell lines and the nasopharyngeal epithelial



Figure 2. Immunohistochemical analysis of CDK3 protein expression in NPC tissue and inflamed tissue. (A-C) Protein immunohistochemistry (original magnification, x400); (A1-C1) the corresponding hematoxylin and eosin staining (original magnification, x400); (A and B) NPC specimens; (C) inflamed nasopharyngeal tissues. CDK3, cyclin-dependent kinase 3; NPC, nasopharyngeal carcinoma.

Table I. Expression of CDK3 protein in NPC and nasopharyn-	-
geal inflammation examined by IHC.	

Туре	Total (n)	Positive	Negative	P-value
NPC	94	63 (67.0)	31 (33.0)	
inflammation	40	5 (12.5)	35 (87.5)	
Total	134	68	66	<0.001

CDK3, cyclin-dependent kinase 3; NPC, nasopharyngeal carcinoma; IHC, immunohistochemistry.

cell line was investigated by western blotting. CDK3 protein expression was detectable in all cell lines. However, compared with the NP-69 nasopharyngeal epithelial cell line, CDK3 was overexpressed in CNE1, CNE2 and 5-8F NPC cell lines (Fig. 1).

CDK3 expression in NPC and inflamed nasopharyngeal tissues by IHC staining. To determine whether the expression level of CDK3 protein is associated with the histological characteristics of NPC, CDK3 expression was examined in samples from 94 cases of NPC and 40 cases of nasopharyngeal inflammation by IHC staining. As shown in Fig. 2, CDK3 was found to be upregulated in NPC (Fig. 2A and B; NPC specimens) compared with in the tissues with nasopharyngeal inflammation (Fig. 2C). There were 63 cases that showed marked or moderate positive CDK3 staining in the 94 NPC samples (67.0%) and only 5 cases out of the 40 cases with nasopharyngeal inflammation (12.5%). The expression levels of CDK3 in NPC and tissues with nasopharyngeal inflammation were significantly different (P<0.001; Table I).

*Expression of CDK3 protein and clinical pathological parameters of NPC*. To determine whether the expression level of CDK3 protein is associated with the clinical pathological parameters of NPC, 94 NPC clinical specimens, which included 18 cases of stage I, 51 cases of stage II, 18 cases of stage III and 7 cases of stage IV NPC, were examined by IHC staining with an antibody against human CDK3. No significant associations were

Clinical nathological		CDK3 expression				
parameter	n	-	+	++	+++	P-value
Degree of infiltration						
T1	38	15	14	8	1	< 0.001
T2	40	13	8	11	8	
T3	13	3	1	4	5	
Τ4	3	0	0	0	3	
Lymph node metastasis						
NO	29	12	8	4	5	< 0.001
N1	52	16	14	14	8	
N2	9	2	1	3	3	
N3	4	1	0	2	1	
TNM clinical stage						
Ι	18	9	6	3	0	< 0.001
II	51	17	15	12	7	
III	18	4	2	6	6	
IV	7	1	0	2	4	
CDK3, cyclin-dependent kinase 3	3; TNM, tumor no	de metastasis.				

Table II. Correlation between CDK3 expression and clinical pathological parameters analyzed by Spearman's correlation test.

observed between CDK3 expression and patient gender (data not shown). However, it was noted that there was a significant correlation between CDK3 protein expression and NPC tumor stage (T stage) (P<0.001), regional lymph nodal metastasis (N stage) (P<0.001) and clinical stage (P<0.001; Table II).

### Discussion

Activation of CDKs induced by overexpression of activator cyclins has been observed in numerous types of human cancer (19). A number of studies have demonstrated that CDK3 was expressed in various human tissues and cell lines (7,12,20). Our previous study also found that CDK3 was overexpressed in glioblastoma tissues and in numerous human cancer cell lines (15). In the present study, CDK3 expression was observed to be elevated significantly in NPC cell lines compared with the nasopharyngeal epithelial cell line, providing further evidence to support the hypothesis that CDK3 is important role NPC tumorigenesis.

CDK3 is an important protein in cell cycle regulation. CDK3 activity occurs early in the G1 phase (21), peaks at mid-G1 (22) and is required for entry into the S phase (13), resulting in increased proliferation, as well as cell transformation (22). This was confirmed in our previous study; for example, knockdown of CDK3 suppressed proliferation and colony formation of T98G glioblastoma cells in soft agar, and suppressed foci formation induced by RasG12V/CDK3/ATF1 in NIH3T3 cells (15). It has been reported that the occurrence of NPC was closely associated with the inactivation of cell cycle-dependence kinase inhibitors, including p16 and p27 (11,23-25) and the overexpression of G1 cyclin D1, G1 cyclin E1 and CDK4 (26,27), suggesting that abnormal expression of cell cycle-associated proteins and protein kinases was directly involved in cell proliferation and malignant transformation of NPC, and therefore was important in the occurrence and development of NPC. In the present study, the expression levels of CDK3 were significantly overexpressed in NPC tissues compared with non-tumor nasopharyngeal epithelium, indicating that CDK3 may be involved in the pathogenesis of NPC. Furthermore, CDK3 expression was correlated significantly with T and N classification and clinical stage, suggesting that the overexpression of CDK3 was associated with aggressive tumor behavior in patients with NPC.

Correct subcellular localization of proteins is critical for their function and for accurate activation of the appropriate pathways by providing physiological context. Aberrant localization of proteins contributes to a number of disorders and diseases, including metabolic, cardiovascular, neurodegenerative diseases and cancer (28). The precise activation of CDKs is mediated through association with a regulatory cyclin subunit, phosphorylation of CDK and subcellular localization (29). In a human prostate cancer cell line, vitamin D-mediated inhibition of cell proliferation was correlated with relocalization of CDK2 from nuclear to cytoplasmic compartments and a significant decrease in CDK2 activity (30,31). Cytoplastic localization of CDK4/6 has functions in the differentiation of pluripotent embryonic cells (32). In the current study, the cytoplasm and nuclei were found to be attached to brown or yellow particles and the majority of the particles were located in the cytoplasm, indicating that CDK3 was primarily localized in the cytoplasm of NPC cells. This finding suggested the possibility that upregulated expression of cytoplasmic CDK3 may provide a selective advantage in the occurrence and progression of NPC. Thus, cytoplastic localization of CDK3 may be beneficial in the development of biomarkers for predicting the progression of NPC patients.

In conclusion, CDK3 expression was characterized in NPC cell lines and clinical tissue specimens by western blotting and IHC, respectively. The expression of CDK3 was elevated in NPC cell lines compared with that in the nasopharyngeal epithelial cell line, and the CDK3 positive expression rate in NPC specimens was significantly higher compared with the inflamed nasopharyngeal tissue specimens. Furthermore, the expression level of CDK3 was markedly correlated with the histological stage of NPC. These results revealed that CDK3 may be a prognostic biomarker in NPC patients. Further studies are required to verify these findings and to clarify the role of CDK3 in NPC.

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