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Original Article

Nuclear expression of CDK6 has a novel prognostic value in patients with nasopharyngeal carcinoma

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Abstract: Aims: Aberrant expression of cyclin-dependent kinase 6 (CDK6) has been reported in several human cancers. However, CDK6 expression in nasopharyngeal carcinoma (NPC) has not been fully investigated. In this study, we examine the expression of CDK6 and analyze the correlation between nuclear expression of CDK6 and clinicopathologic features in NPC patients. Methods: The mRNA level of CDK6 was measured by real-time PCR comparing NPC and nasopharynx tissues. By Western blot, nuclear protein expression of CDK6 was detected between NPC and nasopharynx tissues. Furthermore, the protein expression of CDK6 was examined by immuno-histochemical staining in primary, paraffin-embedded NPC and nasopharynx tissues. Cases with greater than or equal to 10% nuclear expression were scored as being positive for expression of CDK6. The relationship between the nuclear expression levels of CDK6 and clinical features including survival prognosis was analyzed. Results: CDK6 mRNA was markedly elevated in NPC tissues compared to nasopharynx tissues. Nuclear CDK6 protein levels were also observed to be upregulated by immuno-blot assay in NPC tissues compared to nasopharynx tissues. Furthermore, immunohistochemical staining indicated that CDK6 protein was predominantly nuclear in NPC tissues compared to nasopharyngeal carcinoma. CDK6 nuclear expression was shown to be positively correlated with clinical stage (P=0.033) but not associated with other clinical features. Patients with nuclear expression of CDK6 were observed to have poorer overall survival rates than those without nuclear expression of CDK6. In strata analysis, nuclear expression of CDK6 was inversely associated with survival time of NPC patients not only in T1-2, N2-3, M classifications and clinical stage III-IV but also in the non-treated and treated patients who received chemo- or radiotherapy. Finally, it was shown that nuclear expression of CDK6 is an independent factor of unfavorable prognosis in patients with NPC. Conclusions: Our findings demonstrate that CDK6 is overexpressed in NPC and that positive nuclear expression of CDK6 indicates an unfavorable effect on disease progression and poor outcome for NPC patients.

Keywords: CDK6, nasopharyngeal carcinoma, outcome

Introduction

A cyclin-dependent kinase (CDK) is a key regulator of cell cycle transition which is activated upon binding to cyclins. CDK6 as a kinase catalytic subunit of a protein kinase complex, is controlled by regulatory subunits including D-type cyclins. Members of the INK4 family of CDK inhibitors which further regulate Rb activity [1] by phosphorylation leading to the release of E2fs which then activate the transcription of genes required for S-phase entry. Recent evidences have defined CDK6 as an important regulator of stem cell activation and as an essential component of a transcriptional complex suppressing Egr1 in HSCs and LSCs [2], and finally as a regulator of quiescence (defined as the reversible absence of cycling, also called G0) exiting in human hematopoietic stem cells [3].

In the past few years, dysregulated expression of CDK6 has been shown in several tumor types including bladder and pancreatic cancer, T-cell lymphoma, malignant glioma, medulloblastoma, myxofibrosarcomas and B-cell chronic lymphocytic leukemias [4-14] strongly suggesting the involvement of CDK6 in cancer.

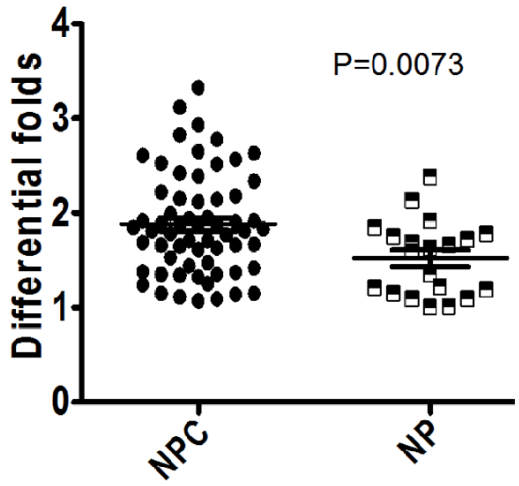

Figure 1. Elevated expression of CDK6 mRNA was shown in NPC NPC: Nasopharyngeal carcinoma; NP: nasopharynx tissues.

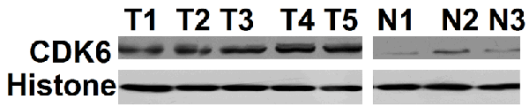

Figure 2. Increased nuclear expression of CDK6 protein was displayed in NPC. T: Nasopharyngeal carcinoma; N: Nasopharynx tissues.

NPC is the most frequent malignant tumor of the nasopharynx. Although it is rare in western populations, it is one of the most common malignancies in Asia, especially in southern China. In a previous study, total protein of CDK6 was observed to be upregulated in nasopharyngeal carcinoma [15, 16]. Furthermore, CDK6 expression is repressed by miR-26 and has been shown to participate in miR-26-mediated the pathogenesis of NPC [17]. These results suggest the significance of CDK6 in NPC pathogenesis. In the current study, the expression of CDK6 in NPC tissues was investigated. The correlation of nuclear protein expression of CDK6 with clinic-pathologic features and patient survival was evaluated. Our results suggest that increased nuclear expression of CDK6 is an unfavorable prognostic factor for NPC patient's progression and survival.

Materials and methods

Sample collection

62 fresh NPC tissues and 20 nasopharynx tissues were collected from the People's Hospital of Zhongshan City, China, at the time of diagnosis. All fresh samples were preserved immediately in liquid nitrogen. 113 undifferentiated NPC and 31 nasopharynx paraffin embedded samples were obtained from patients ranging in age from 21 to 76 years at the People's Hospital of Zhongshan City. Amongst these patients, 5 patients had been treated with chemotherapy alone, 14 with radiotherapy alone and 103 with combined treatment of radiotherapy and chemotherapy. Ten patients did not accept any treatment. To use these clinical materials for research purposes, we obtained prior informed patients' consent and the approval from the Hospital Ethics Committee. All specimens had confirmed pathological diagnosis and were staged according to the 1997 NPC staging system of the UICC.

Real-time PCR

CDK6 mRNA was measured by Real-time PCR in 62 fresh NPC tissues and 20 nasopharynx tissues based on the SYBR Premix Ex Taq (Takara, Shiga, Japan) method as described previously [17]. The sense and anti-sense primers of CDK6 were 5'-TCCCCAGAGTCTGATTACCT-3' and 5'-ACGATTACATAGCCTCTGCC-3' respectively. The *ACTB* gene was used as internal control.

Nuclear protein assay of CDK6 by western blot assay

Nuclear protein was extracted from 5 fresh NPC samples and 3 fresh nasopharynx samples according to the instructions of the nuclear protein extraction kit (Gaiji Inc, Nanjing, China). Protein lysates were resolved on 10% SDS polyacrylamide gel, electro-transferred to poly-vinylidene fluoride membranes (Invitrogen, Inc. Carlsbad, CA, USA), and blocked in 5% nonfat dry milk in Tris-buffered saline, pH 7.5 (100 mM NaCl, 50 mm Tris and 0.1% Tween-20). Membranes were immuno-blotted overnight at 4°C with an CDK6 antibody at a dilution of 1:200 (Cell Signaling Technology Inc, USA) and a histone3 antibody at a dilution of 1:1000 (Cell Signaling Technology Inc, USA), followed by their respective horseradish peroxidase (HRP)-conjugated secondary antibodies. Signals were detected by enhanced chemi-luminescence (Pierce, Rockford, IL, USA).

Immunohistochemistry

Immuno-histochemistry was performed as described [18] previously with a rabbit anti-

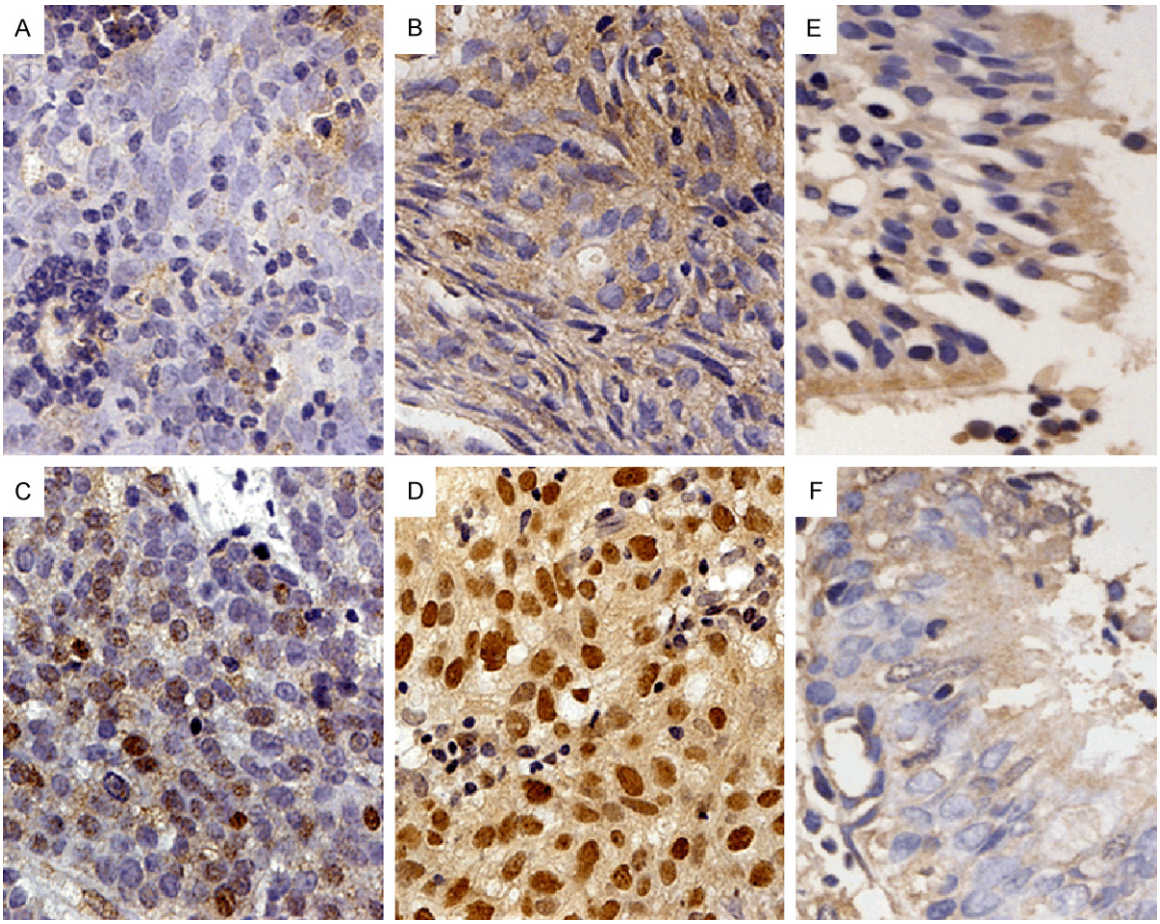

Figure 3. Expression of CDK6 protein in the cytoplasm and nuclei of NPC samples (original magnification: ×400). A, B: CDK6 protein expression in cytoplasm of NPC tissues; C, D: Positive CDK6 protein expression in cellular nucleus of NPC tissues: E, F: CDK6 protein expression in cytoplasm of nasopharynx tissues.

Table 1. Increased nuclear expression of CDK6 protein in NPC

Tissue	N	Nuclear CDK6 expression		*P* value
		Positive	Negative	
NPC	113	49	64	0.000
Nasopharynx	31	4	37	

human CDK6 polyclonal antibody at concentration of 1:100 (Santa Cruz Biotechnology, USA) (Santa Cruz Biotechnology, USA). Sections were visualized with DAB and counter stained with hematoxylin, mounted in neutral gum and analyzed using a bright field microscope.

Evaluation of staining

The stained tissue sections were reviewed separately by two pathologists blinded to the clinical parameters and evaluated for the presence of nuclear staining. Tumor cells with nuclear staining of more than or equal to 10% were considered as positive for nuclear expression. Less than 10% staining was regarded as negative.

Statistical analyses

All statistical analyses were carried out using SPSS 13.0. A T-test was used to analyze the differential expression of CDK6 mRNA between NPC and nasopharynx tissues. The χ^2 test was utilized to analyze the relationship between CDK6 nuclear expression and clinico-pathologic characteristics. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. The significance of various variables in survival was analyzed using multivariate Cox proportional hazards model. A *P*-value of less than 0.05 was considered statistically significant.

Table 2. Correlation between the clinicopathologic characteristics and nuclear expression of CDK6 protein in NPC

Characteristics	N	Nuclear expression of CDK6 (%)		*P*
		No	Yes	
Gender				
Male	76	45	31	
Female	37	19	18	0.544
Age (y)				
≥50	55	35	23	
<50	58	34	21	1.000
Smoking				
Yes	19	10	9	
No	94	54	40	0.801
Family tumor history				
Yes	6	2	4	
No	107	62	45	0.400
T classification				
T_1-T_2	83	49	34	
T_3-T_4	30	15	15	0.400
N classification				
N_0-N_1	68	44	24	
N_2-N_3	45	20	25	0.052
Distant metastasis				
Yes	9	4	5	
No	104	60	44	0.498
TNM Clinical stage				
I~II	46	32	14	
III~IV	67	32	35	0.033

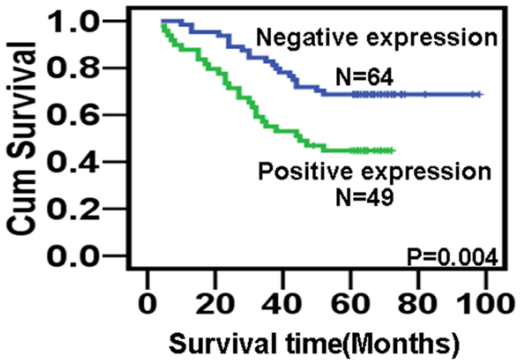

Figure 4. Nuclear expression of CDK6 protein was unfavorable for NPC patients overall survival time.

Results

CDK6 mRNA was highly elevated in NPC

Real-time PCR was used to measure the expression of CDK6 mRNA transcripts between 62 fresh NPC and 31 nasopharynx tissues. The results indicated that CDK6 mRNA level was significantly elevated in NPC tissues (P=0.0073) (**Figure 1**).

Nuclear protein expression of CDK6 in NPC

To further explore the role of CDK6, nuclear expression of CDK6 protein was examined in NPC and nasopharynx tissues by western blot analysis. The results indicated that CDK6 expression was significantly increased in the nucleus of NPC tissues compared to those of nasopharynx tissues (**Figure 2**).

Immunohistochemistry of CDK6 in NPC tissues

Further, we examined the expression levels and subcellular localization of CDK6 protein in 113 NPC samples and 31 nasopharynx tissues using immuno-histochemical staining (**Figure 3**). It was found that specific CDK6 protein was stained in the nuclei and cytoplasm of tumor cells. Furthermore, it was observed that 43.4% (49/113) (**Tables 1** and **2**) cases showed positive nuclear expression of CDK6 protein. However, in nasopharynx tissues, only 12.9% (4/31) cases indicated positive nuclear expression of CDK6 protein (P<0.001) (**Table 1**).

Correlation between clinico-pathological feature and CDK6 nuclear expression in NPC patients

The correlation between CDK6 expression and clinical characteristics was analyzed. As shown in **Table 2**, a significant relationship between CDK6 nuclear expression with patient age, sex, smoking, T classification, N classification or distant metastasis (M classification) in 113 NPC cases was not observed but the nuclear expression of CDK6 was shown to positively correlate with clinical stage (I-II vs. III-IV) (P=0.033) in NPC patients. Furthermore, an association of CDK6 nuclear expression with N classification in N2-N3 compared to N0-N1 samples (P= 0.052) was also found.

CDK6 nuclear expression negatively correlates with overall survival time of NPC

To assess the prognostic value of CDK6 expression for NPC, Kaplan-Meier analysis with the log-rank test was used to analyze the association between the levels of CDK6 expression and patient survival. The level of CDK6 nuclear protein expression was shown to be negatively

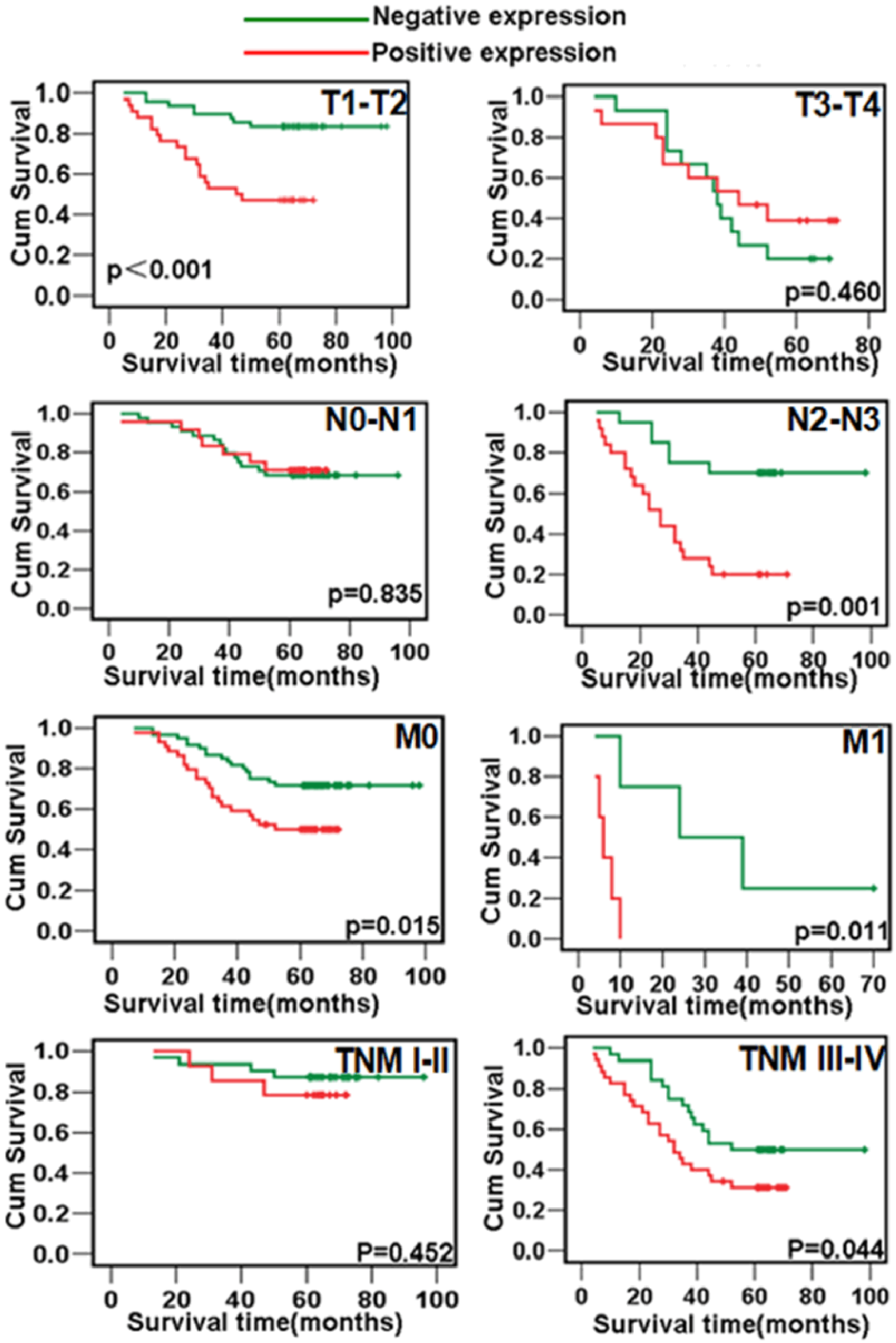
Negative expression
Positive expression
T1-T2
p<0.001
T3-T4
p=0.460
N0-N1
p=0.835
N2-N3
p=0.001
M0
p=0.015
M1
p=0.011
TNM I-II
P=0.452
TNM III-IV
P=0.044
Cum Survival
Survival time(months)

Figure 5. The correlation of CDK6 expression with NPC patients' survival time in strata analysis in T, N, M and TNM classification. CDK6 protein expression was significantly associated with survival time for NPC patients in T1-2, N2-3, M0 and M1 classification and clinical stage III-IV, but did not correlate with T3-4 classification, N0-1 classification, and clinical stage I-II. Patients with nuclear expression of CDK6 protein had shorter survival times in T1-2, N2-3, M0 and M1 classification, and clinical stage III-IV.

correlated with the overall survival time of NPC patients. Patients with nuclear expression also had worse prognosis than those with negative nuclear expression of CDK6 (**Figure 4**) (P= 0.004).

Nuclear expression of CDK6 is inversely associated with survival time of NPC patients for T1-2, N2-3, M classification and clinical stage III-IV

The correlation between CDK6 nuclear expression and NPC patient prognosis by strata analysis was performed against T and N classification and clinical stage. The results indicated that CDK6 protein nuclear expression was significantly associated with survival time for NPC patients in T1-2 classification (P<0.001), N2-3 classification (P=0.001), M0 classification (P= 0.015), and M1 classification (P=0.011) (**Figure 5**). Furthermore, patients with nuclear expression had worse prognosis than those with negative nuclear expression of CDK6.

Nuclear expression of CDK6 is unfavorable for radiotherapy and chemotherapy treated NPC patients

The association of nuclear CDK6 expression and the treatment with chemo- or radiotherapy for NPC patient survival was investigated. A significant correlation of CDK6 nuclear expression with NPC prognosis in both chemotherapy (P=0.019) and radiotherapy treatment groups (P=0.009) was observed but not in the non-treated group (**Figure 6**). Patients with nuclear expression had poorer prognosis than those with negative nuclear expression of CDK6 after respective treatment with chemotherapy and radiotherapy.

Nuclear expression of CDK6 as an independent prognosis factor for NPC patients

To investigate the potential of CDK6 nuclear expression as an independent prognostic marker, the multivariate Cox proportional hazards model was used to analyze the significance of variables on survival. Univariate analyses indicated that T, N, M classifications, clinical stages and radiotherapy were also significantly associated with patient survival (where P=0.001, P=0.001, P=0.001, P<0.001, and P=0.002 respectively). Further, multivariate analysis of CDK6 protein expression levels adjusted for radiotherapy, T classification, N classification, M classification, and clinical stages of NPC patients showed that CDK6 nuclear expression was an independent prognostic marker for NPC patients (P=0.003) (**Tables 2** and **3**).

Discussion

Dysregulation of cell cycle control is a basic process in tumor growth which is in almost all types of tumors. In this study, we focused on CDK6 protein expression to determine its potential role in NPC. CDK6 has been reported to co-operate with cyclin D and CDK4 in driving cell cycle progression from G1 to S phase through the phosphorylation and subsequent inactivation of Rb protein. Its abnormal expression has been detected in a few tumors and suggested it could promote disease progression and poor prognosis in these patients. The expression pattern of CDK6 and its correlation with clinical features, survival and prognosis has still to be fully determined in NPC.

In this study, we firstly observed that CDK6 mRNA expression was markedly increased in NPC tissues compared to nasopharynx tissues. This data were consistent with that of Baba and Luo, in esophageal squamous cell carcinoma and NPC [15, 19] supporting the oncogenic role of CDK6 in NPC.

In a previous study, CDK6 had been reported to bind CDK4 and CCND1, and form a nuclear complex which induced cell cycle progression [20] and promoted cell growth in tumors [21, 22]. Mahony *et al*. showed the presence of CDK6 in the cytoplasm and nucleus of T-cells but only nuclear CDK6 was active and showed the ability to phosphorylate Rb [6]. These studies suggest the significance of CDK6 nuclear expression in tumor pathogenesis. Furthermore, we observed that the nuclear expression of

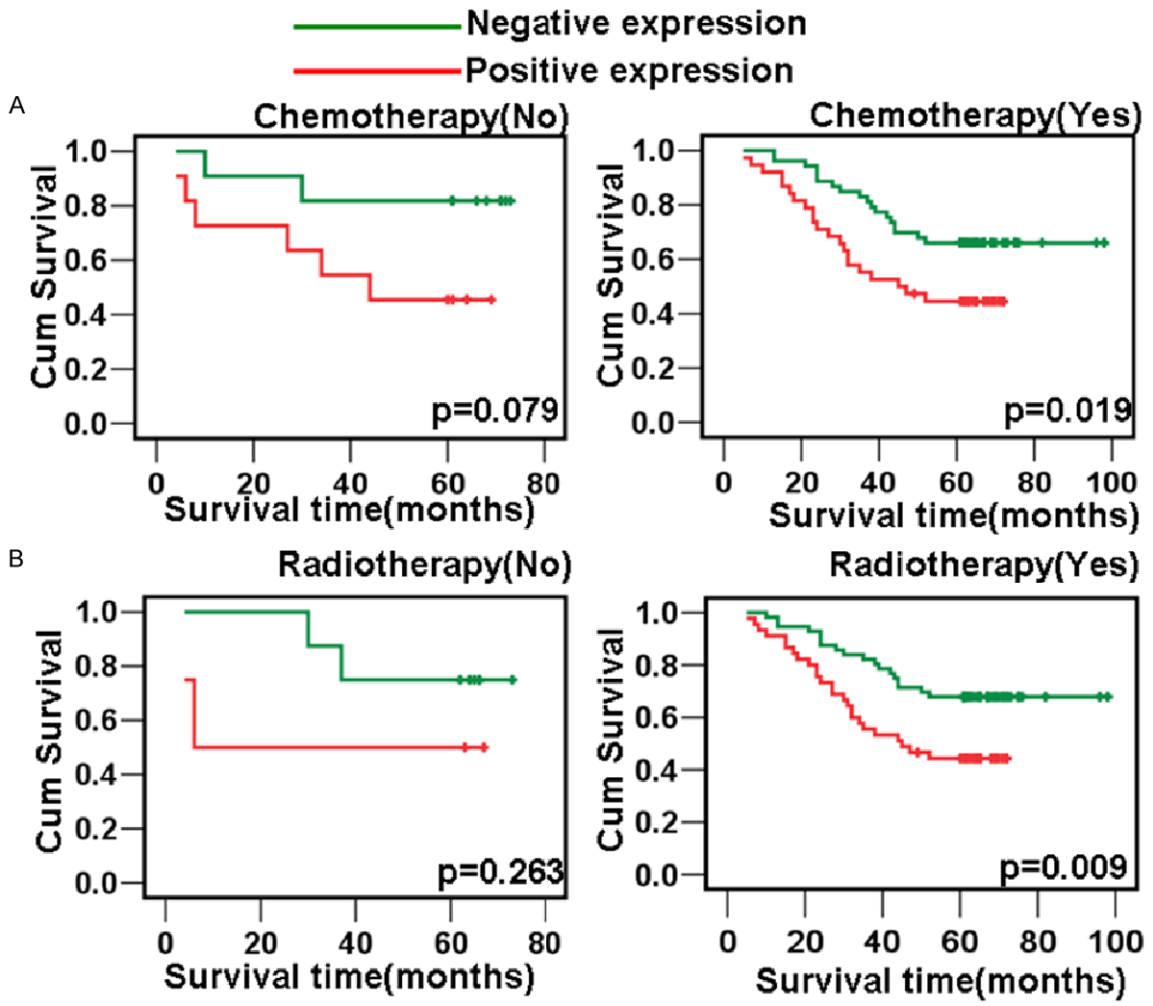

Figure 6. Nuclear expression of CDK6 was unfavorable for radiotherapy and chemotherapy for NPC patients. A. Patients with nuclear expression had poorer prognosis than those with negative nuclear expression of CDK6 after treatment with radiotherapy. B. Patients with nuclear expression had poorer prognosis than those with negative nuclear expression of CDK6 after treatment with chemotherapy.

CDK6 protein was increased compared to nasopharynx tissues by immune-blot assay. In agreement with this data, it was found that nuclear CDK6 protein expression was markedly increased in the cell nucleus of NPC tissues compared to the nasopharynx by immuno-histochemical staining. This finding was consistent with previous reports in other tumors types [4-9]. We further investigated the correlation of CDK6 nuclear expression with clinical features of NPC patients. It was shown that CDK6 nuclear expression was not correlated with patient age, sex, smoking, tumor size and distant metastasis but was positively correlated with clinical stage. Furthermore, we observed a positive association of CDK6 nuclear expression with lymph node metastasis in N2-N3 compared to N0-N1 samples. Our results suggested that CDK6 nuclear expression promotes NPC progression and further supported CDK6 as an oncogene in tumors.

In previous studies, overexpression of CDK6 has been shown to significantly correlate with poor prognosis in patients with myxofibrosarcomas and medulloblastoma [8-10]. However, these investigations did not present the significance of CDK6 nuclear expression in tumors. In this study, CDK6 nuclear expression was strongly unfavorable for the prognosis of NPC patients. Patients with CDK6 expression had an overall shorter survival time than those of patients with negative nuclear CDK6 expression. These results are similar to those of Tsai,

Table 3. Summary of univariate and multivariate Cox regression analysis of overall survival duration

Parameter	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	P	HR	95% CI
Gender						
Male vs. female	0.805	1.080	0.585-1.996			
Age						
≥50 vs. <50 years	0.447	1.249	0.704-2.216			
Family tumor history						
Yes vs. No	0.679	1.280	0.397-4.127			
Smoking						
Yes vs. No	0.894	1.053	0.492-2.253			
Chemotherapy						
Yes vs. No	0.746	1.134	0.530-2.247			
Radiotherapy						
Yes vs. No	0.002	0.300	0.140-0.645	0.019	0.361	0.154-0.844
T classification						
T_1-T_2 vs. T_3-T_4	0.001	2.781	1.559-4.961	0.110	1.768	0.880-3.553
N classification						
N_0-N_1 vs. N_2-N_3	0.001	2.586	1.451-4.606	0.102	1.861	0.883-3.921
M classification						
M_0 vs. M_1	0.001	7.717	3.310-15.558	0.000	7.258	2.908-18.111
Clinical stage						
I-II vs. III-IV	0.000	5.512	2.463-12.336	0.244	1.929	0.639-5.820
Nuclear expression of CDK6						
Positive expression vs. Negative expression	0.006	2.268	1.271-4.048	0.003	2.540	1.369-4.713

Hayette and Mendrzyk *et al.* reports in tumors [8-10]. In addition, the correlation of CDK6 nuclear expression with survival prognosis of NPC patients was further evaluated by a strata analysis against different T, N, and M classifications and clinical stages. The results indicated that nuclear expression of CDK6 in T1-T2, N2-3, M0, M1, and TNM classification promoted poor prognosis and led to shorter overall survival time. These results described above suggested CDK6 nuclear expression as a preferable biomarker for evaluating the prognosis of NPC patients.

CDK6 acts as an oncogenic factor promoting cell growth in some tumor pathogeneses. Nuclear expression of CDK6 may also be associated with resistance to radiotherapy and chemotherapy, reducing overall survival time for NPC patients. Consistent with our findings, patients with nuclear expression of CDK6 protein had an overall shorter survival time suggesting CDK6 nuclear expression might participate in resistance to radiotherapy and chemotherapy.

In previous studies, CDK6 expression as a prognosis marker has been indicated in myxofibrosarcomas and medulloblastoma [8, 10]. In this investigation, we explored the possibility of CDK6 nuclear expression as an independent prognostic factor. Based on univariate analysis, overall patient survival is inversely proportional to T/N/M classification and clinical stage, and CDK6 nuclear expression, and positively correlated with radiotherapy. Furthermore, nuclear expression of CDK6 protein represents an independent prognostic marker of overall survival based on multivariate analysis for NPC patients regardless of disease status.

In summary, our study demonstrates that nuclear expression of CDK6 is involved in the clinical progression and poor prognosis of NPC patients. Furthermore, our data suggest that CDK6 can serve as a potential independent outcome biomarker for NPC patients.

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Disclosure of conflict of interest

None.

Abbreviations

CDK6, Cyclin-dependent kinase; NPC, nasopharyngeal carcinoma; EBV, Epstein-Barr virus; NP, nasopharynx specimen.

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