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Original Article

Elevated nuclear CDK6 is associated with an unfavorable prognosis in lung adenocarcinoma patients

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Abstract: Aims: It has been recently reported that cyclin-dependent kinase 6 (CDK6) is abnormally expressed in several human malignant tumors; however, CDK6 nuclear expression has not been fully studied in lung adenocarcinoma. We determined the correlation between the level of CDK6 expression and clinicopathologic data in lung adenocarcinoma patients, including survival. Methods and results: The level of expression of CDK6 protein was detected by immunohistochemical analysis in 143 paraffin-embedded lung adenocarcinoma tissues and 35 normal paraffin-embedded lung tissues. The data showed that overexpression of nuclear CDK6 protein was present in lung adenocarcinoma. Further, we analyzed the correlation between the levels of nuclear CDK6 expression and clinical features, including survival prognosis. Higher expression of nuclear CDK6 protein was significantly associated with N stage (P=0.012), clinical stage (P=0.008), and degree of differentiation (P=0.015), but there was no association with T stage (P=0.472) or distant metastasis (P=0.163). Increased CDK6 expression was associated with poorer overall survival rates than low expression of CDK6 (P=0.004). Multivariate analysis indicated that high expression of nuclear CDK6 was an independent prognostic indicator of patient survival. Conclusions: Our findings demonstrate that nuclear CDK6 protein is overexpressed and plays a detrimental role in disease progression and poor outcome with lung adenocarcinoma patients.

Keywords: CDK6, lung adenocarcinoma, disease progression, prognosis

Introduction

Lung adenocarcinoma is the leading cause of cancer-related deaths worldwide and morbidity is on the rise [1]. Of lung adenocarcinomas, 85% are non-small cell lung carcinoma (NSCLC) [2]. Although a number of studies have identified new prognostic and predictive molecular markers to improve treatment stratification and overall survival, such as EGFR, CD66b, BIRC6, SIRT1, and miR-155 [1-4]. In the past two decades, the 5-year survival rate for lung adenocarcinoma has remained at 15% [5].

Carcinogenesis is thought to be caused by dysregulation of a cell cycle mechanism. A family of serine/threonine protein kinases, cyclin-dependent kinases (CDKs), are involved in the cell cycle, transcription, translation, neurogenesis, and apoptosis [6]. Deregulation of CDKs is directly linked to oncogenesis. CDK6 is a member of CDKs, and is controlled by regulatory subunits, including the CDK6-cyclin D complex, to exert its catalytic activity. CDK6 phosphorylates the tumor suppressor retinoblastoma protein (Rb) to release transcriptional repression of E2F-dependent genes, then drives the cells from cell cycle G1 phase-to-S phase, and negatively regulates cell differentiation [7]. CDK6 also has a non-canonical kinase-independent function as an important regulator of transcription in at least two distinct ways (as a partner with STAT3 and D-type cyclins to induce p16 INK4a expression or together with the AP-1 transcription factor, c-JUN, when it up-regulates VEGF-A) [8].

In previous studies, up-regulated CDK6 has been shown to be associated with the development of several types of cancers, including bladder, pancreatic, T-cell lymphoma, endometrial cancer, medulloblastoma, myxofibrosarcoma, and oral cancer, breast cancer [9-16]. In the current study, CDK6 was also shown to be

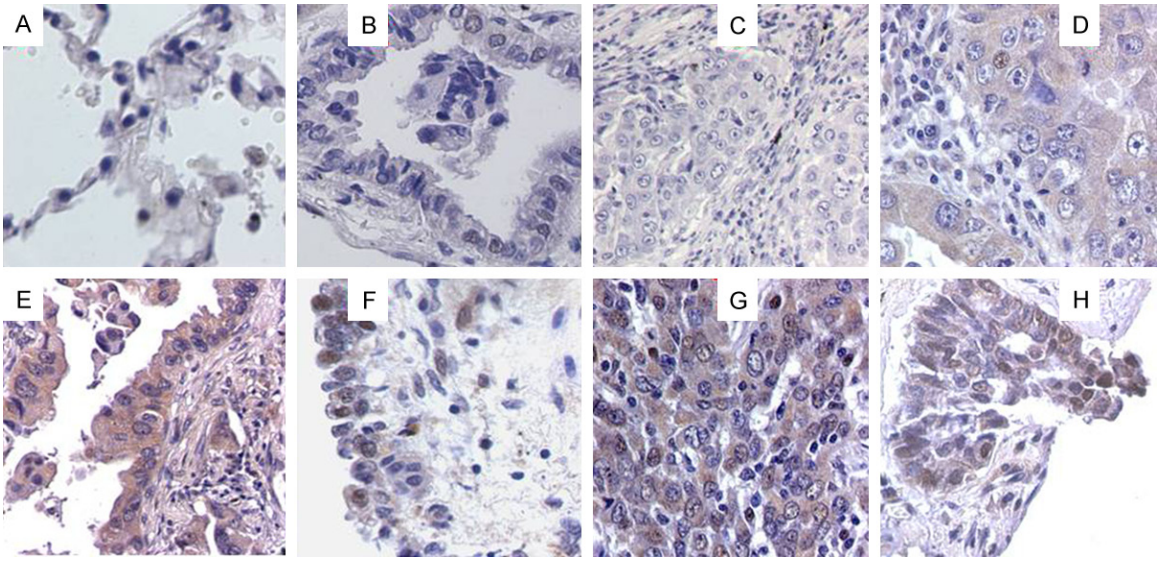

Figure 1. CDK6 expression in normal lung and lung adenocarcinoma tissues samples (original magnification: ×400). A and B: Negative expression of CDK6 protein in normal lung tissues. C: Negative expression of CDK6 in lung adenocarcinoma; D and E: Predominant cytoplasm expression of CDK6 in lung adenocarcinoma. F-H: Nuclear and cytoplasmic expression of CDK6 in lung adenocarcinoma. C and D: High differentiated lung adenocarcinoma; E and F: Medium differentiated lung adenocarcinoma; G and H: Low differentiated lung adenocarcinoma.

Table 1. Elevated expression of nuclear CDK6 protein in lung adenocarcinoma

	N	Nuclear CDK6 expression		*P* value
		High	Low	
Cancer	143	73	70	0.003*
Normal	35	8	27	

*, Statistically significant.

overexpressed in lung adenocarcinoma cells [16]; however, the correlation between expression of nuclear CDK6 protein with clinic-pathologic features and patient survival has not been evaluated. Our results indicate that increased expression of nuclear CDK6 is an unfavorable independent prognostic factor for lung adenocarcinoma progression and patient survival.

Materials and methods

Sample collection

143 lung adenocarcinoma and 35 lung paraffin-embedded samples were obtained from patients ranging in age from 19-75 years at the People's Hospital of Zhongshan City. To use these clinical materials for research purposes, we obtained prior informed patient consent and approval from the Hospital Ethics Committee. All specimens had a confirmed pathologic diagnosis and were staged according to the 2009 lung cancer staging system of the UICC.

Immunohistochemistry

According to standard protocols, the lung adenocarcinoma paraffin sections (3 μm) were deparaffinized in 100% xylene and rehydrated in descending ethanol series (100%, 90%, 80%, and 70% ethanol). Heat-induced antigen retrieval was performed in 10 mM citrate buffer for 2 min at 100°C. A peroxidase blocking reagent containing 3% hydrogen peroxide and serum to block endogenous peroxidase activity, and non-specific antigen was followed by incubation with a rabbit anti-human CDK6 polyclonal antibody at a concentration of 1:100 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C overnight. The sections were visualized with DAB and counterstained 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. Positive expression was designated at >20% of cells showing staining, <20% staining was regarded as negative expression.

Table 2. Correlation between the clinicopathologic characteristics and expression of CDK6 in lung adenocarcinoma

Characteristics	n	CDK6 expression		*P* value
		High	Low	
Age (y)				
<60	75	39 (52.0%)	36 (48.0%)	0.811
≥60	68	34 (50.0%)	34 (50.0%)	
Gender				
Male	105	59 (56.2%)	46 (43.8%)	0.041
Female	38	14 (36.8%)	24 (63.2%)	
Degree of differentiation				
High	24	6 (25.0%)	18 (75.0%)	0.015
Medium	39	20 (51.3%)	19 (48.7%)	
Low	80	47 (58.8%)	33 (41.2%)	
TNM classification				
I-II	81	34 (42.0%)	47 (58.0%)	0.008
III-IV	62	39 (62.9%)	23 (37.1%)	
T classification				
T1-T2	115	57 (49.6%)	58 (50.4%)	0.472
T3-T4	28	16 (57.1%)	12 (42.9%)	
N classification				
N0-N1	85	36 (42.4%)	49 (57.6%)	0.012*
N2-N3	58	37 (63.8%)	21 (36.2%)	
Distant metastasis				
Yes	2	2 (100.0%)	0 (0.0%)	0.163
No	141	71 (50.4%)	70 (49.6%)	

*, Statistically significant.

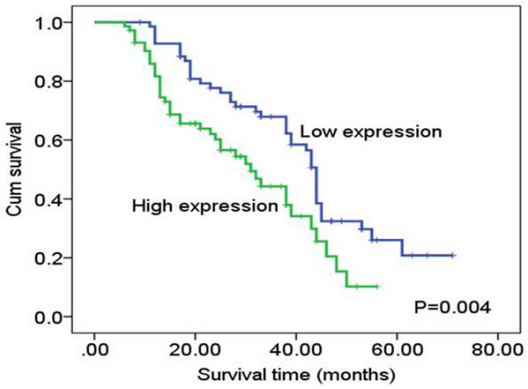

Figure 2. High expression of nuclear CDK6 protein was unfavorable for lung adenocarcinoma patient overall survival time.

Statistical analyses

All statistical analyses were carried out using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). A chi-square test was used to analyze the correlation between the level of CDK6 nuclear expression and clinicopathologic parameters of lung adenocarcinoma. The association between the level of nuclear CDK6 and survival was examined by Kaplan-Meier analysis with the log-rank test. Multivariate survival analysis was performed using Cox proportional hazards regression model. A *P* value <0.05 was considered statistically significant.

Results

Immunohistochemistry of CDK6 in lung adenocarcinoma tissues

We examined the level of nuclear expression and subcellular localization of CDK6 protein in 143 archived paraffin-embedded lung adenocarcinoma samples and 35 normal lung samples using immunohistochemical staining. Specific CDK6 protein staining was detected in the nuclei and cytoplasm of non-cancerous and malignant epithelial cells (**Figure 1**). Furthermore, the expression of nuclear CDK6 was significantly increased in lung adenocarcinoma tissues (73/143 [51.0%]) compared to non-cancerous tissues (8/27 [23%]; **Table 1**).

Correlation between CDK6 expression and clinicopathologic parameters in lung adenocarcinoma patients

The correlations between CDK6 expression and clinicopathologic parameters in lung adenocarcinoma patients were summarized. As shown in **Table 2**, a significant relationship between CDK6 nuclear expression with patient age, gender, T classification, or distant metastasis (M classification) in 143 lung adenocarcinoma was not observed, but high nuclear CDK6 expression was associated with disease progression, including TNM stage (*P*=0.008), degree of differentiation (*P*=0.015), and N classification (*P*=0.012; **Table 2**). These results demonstrated that the change in nuclear CDK6 expression reflects symptoms and disease progression.

High expression of nuclear CDK6 correlates with poor overall survival

To evaluate the prognostic value of CDK6 expression in lung adenocarcinoma tissues, we

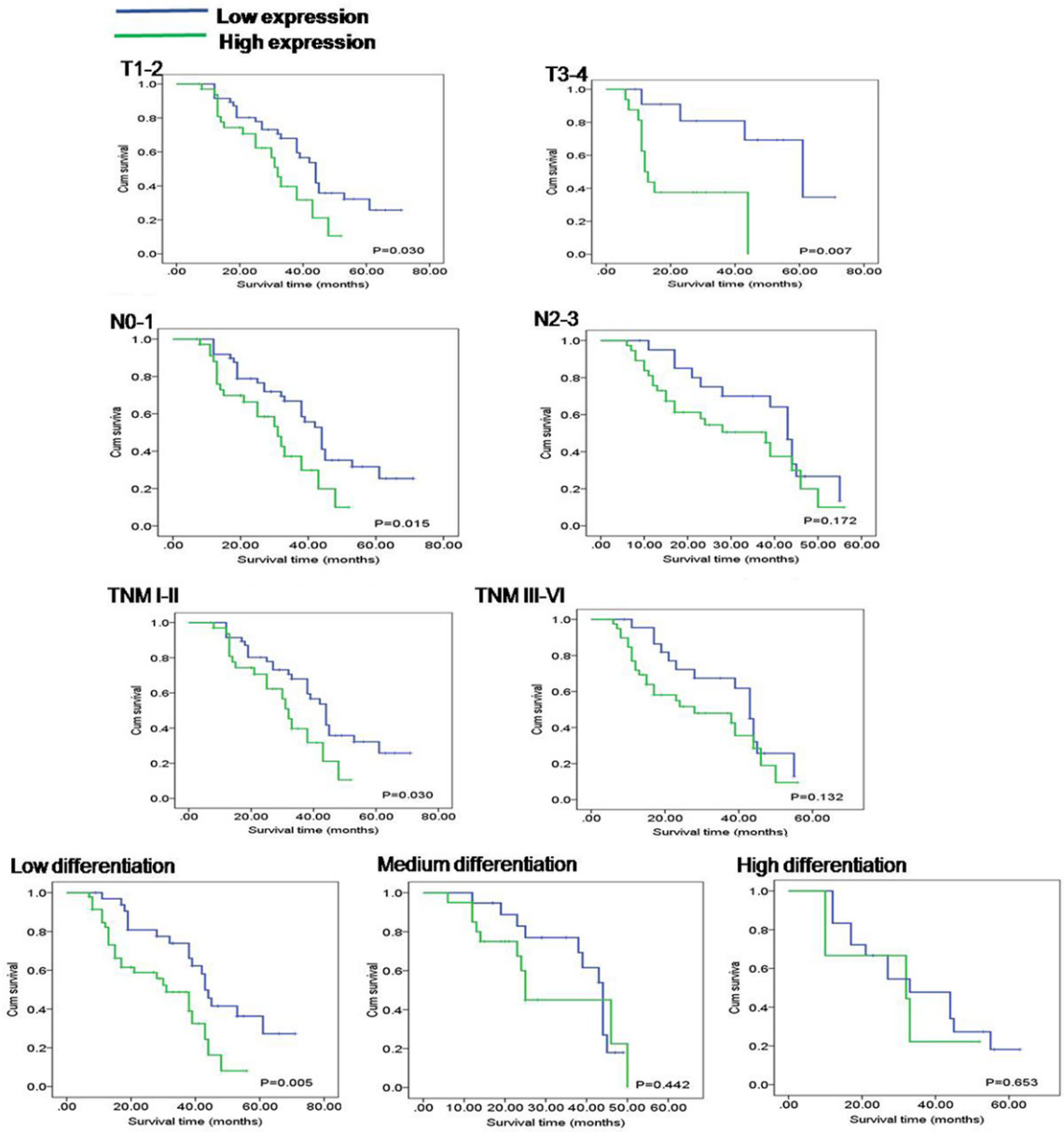

Figure 3. The correlation between CDK6 expression and lung adenocarcinoma patient survival time based on strata analysis of T, N, and TNM classification and differentiation. CDK6 protein expression was significantly associated with survival time for NPC patients in T1-2, T3-4, and N0-1 classification, low differentiation, and clinical stages I-II, but did not correlate with T3-4 classification, N2-3 classification, and clinical stages III-IV. Patients with nuclear expression of CDK6 protein had shorter survival times in T1-2, T3-4, and N0-1 classification, low differentiation, and clinical stages I-II.

used Kaplan-Meier analysis with the log-rank test to analyze the association between the levels of nuclear CDK6 expression and patient survival. The level of CDK6 nuclear protein expression was shown to be negatively associated with the overall survival time of lung adenocarcinoma patients. Patients with high nuclear CDK6 expression had a worse prognosis than patients with low nuclear expression of CDK6 (**Figure 2**; P=0.004).

Increased CDK6 nuclear expression was inversely associated with survival time of lung adenocarcinoma patients for T classification, N0-1 classification, low differentiation, and clinical stage I-II

The correlation between CDK6 nuclear expression and lung adenocarcinoma patient prognosis by strata analysis was performed against T and N classifications, clinical stage, and differ-

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

	Univariate analysis			Multivariate analysis		
Parameter	*P* value	HR	95% CI	*P* value	HR	95% CI
Age						
≥60 versus <60 years	0.162	1.362	0.883-2.102			
Gender						
Male versus female	0.072	1.604	0.959-2.684	0.201	1.410	0.832-2.389
Degree of differentiation						
High versus Medium versus Low	0.798	1.036	0.790-1.358			
TNM classification						
I-II versus III-IV	0.144	1.380	0.896-2.127			
T classification						
T1-T2 versus T3-T4	0.928	0.974	0.555-1.710			
N classification						
N0-N1 versus N2-N3	0.334	1.240	0.802-1.916			
M classification						
M0 versus M1	0.916	0.899	0.124-6.429			
Expression of CDK6						
High versus low expression	0.005*	1.895	1.215-2.956	0.014*	1.772	1.125-2.791

*, Statistically significant.

entiation. We found that high expression of nuclear CDK6 protein was significantly associated with survival time for lung adenocarcinoma patients in T1-2, T3-4 (*P*=0.030, *P*=0.007), and N0-1 classification (*P*=0.015), clinical stages I-II (*P*=0.030), and low differentiation (*P*=0.005; **Figure 3**), while patients with N2-3 stage, medium and high differentiation tumors, and clinical stages III-IV showed no association between CDK4 nuclear expression and prognosis. Furthermore, lung adenocarcinoma patients with high nuclear expression had a worse prognosis than patients with low nuclear expression of CDK6.

High expression of nuclear CDK6 is an independent prognosis factor for lung adenocarcinoma patients

We used the univariate and multivariate Cox proportional hazards model to analyze the significance of various variables in survival to investigate the potential high expression of nuclear CDK6 is an independent prognosis factor. Univariate and multivariate analyses both suggested that CDK6 nuclear expression were significantly associated with patient survival (*P*=0.005 and *P*=0.014). High expression of nuclear CDK6 tended to be an independent prognostic marker for lung adenocarcinoma patients (**Table 3**).

Discussion

Cell cycle deregulation is a common process for the development of all cancers. CDK6 is an important regulator during the G1/S cell cycle transition. CDK6 co-operates with cyclin D and CDK4 to phosphorylate Rb protein, subsequently leading to the release of E2F transcriptional factors. Aberrant expression of CDK6 protein has been observed in many tumors, suggesting that aberrant expression of CDK6 protein promotes disease progression and poor prognosis in tumor patients. In the current study it concentrated on CDK6 expression to confirm a potential role in lung adenocarcinoma [16]. Nevertheless, the correlation between nuclear expression of CDK6 and clinical features, survival, and prognosis is unclear in patients with lung adenocarcinoma.

We showed that CDK6 is expressed in the nucleus and cytoplasm of lung adenocarcinoma and lung tissues by immunohistochemistry. Our results showed that CDK6 nuclear expression is specifically elevated in high differentiated lung adenocarcinoma samples compared to lung tissues. The data were consistent with previous investigations involving pancreatic, lymphoma, medulloblastoma, leukemia and prostate cancers [10, 11, 13, 17, 18]; however, these studies focused on total CDK6 protein.

Our studies indicate that increased nuclear expression of CDK6 might promote the pathogenesis of lung adenocarcinoma.

Further, we analyzed the association between the level of CDK6 nuclear expression and clinical features of lung adenocarcinoma patients. High expression of nuclear CDK6 was not associated with patient age, gender, tumor size, or distant metastasis, but the expression of nuclear CDK6 was positively correlated with lymph node metastasis, clinical stage, and degree of differentiation. This result was in agreement with the findings from a study of myxofibrosarcoma [14], but not consistent with the study conducted by Sopee et al. [7] in head and neck squamous cell carcinoma patients. Nuclear CDK6 expression was significantly correlated with T classification. Our study suggested that expression of nuclear CDK6 protein promotes the clinical progression of lung adenocarcinoma.

Subsequently, we proved that high expression of nuclear CDK6 protein in lung adenocarcinoma is inversely correlated with overall survival time, and indicated that high nuclear expression of CDK6 is a significant clinical biomarker for lung adenocarcinoma prognosis. Our data was similar to the study conducted by Mendrzyk et al. [13] in medulloblastoma patients; however, the investigation did not present the significance of CDK6 nuclear expression in tumors. We found that high expression of nuclear CDK6 was inversely associated with survival time in stages T1-T2 and T3-T4, clinical stages I-II, stage N0-1, and low differentiation, suggesting that elevated nuclear CDK6 expression may function in promoting cell proliferation and clinical progression in early stage of disease more than advanced stage in lung adenocarcinoma.

Finally, we evaluated whether or not CDK6 nuclear expression was an independent prognostic factor for lung adenocarcinoma. The univariate and multivariate analyses showed whether or not overall survival is correlated with CDK6 nuclear expression. In spite of patient disease status, the result indicated that high nuclear expression of CDK6 protein is an independent predictor of prognosis for lung adenocarcinoma patients. The result was similar to a study involving nasopharyngeal carcinoma, in which nuclear expression of CDK6 protein represented an independent prognostic marker of overall survival [19].

Together, these results demonstrated that higher expression of nuclear CDK6 may be involved in the clinical progression and poor prognosis of lung adenocarcinoma patients. Higher expression of nuclear CDK6 could also be considered as a potential independent prognostic factor for lung adenocarcinoma.

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Disclosure of conflict of interest

None.

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