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Original Article

Elevated nuclear CCND1 expression confers an unfavorable prognosis for early *stage* lung adenocarcinoma patients

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Abstract: Purposes: To examine the expression pattern of CCND1 and analyze the correlation of its nuclear expression with clinicopathologic features and prognosis in lung adenocarcinoma. Methods: CCND1 mRNA and protein levels in lung adenocarcinoma tissues were examined. The relationship between nuclear CCND1 protein expression and clinical features including survival prognosis was analyzed. Results: CCND1 mRNA levels were markedly increased in lung adenocarcinoma (P=0.0019). Western blot analysis confirmed increased nuclear CCND1 protein expression in lung adenocarcinoma specimens. Immunohistochemistry analysis confirmed that CCND1 protein was predominantly nuclear localized in lung adenocarcinoma cells and significantly elevated relative to normal lung tissues (P<0.001). Furthermore, high levels of nuclear CCND1 were positively correlated with clinical stage (P=0.026). Patients with nuclear CCND1 expression had a significantly shorter overall survival time than did patients with low expression. Interestingly, nuclear CCND1 expression in clinical stage I+II, but not clinical stage III, was shown associated with poor prognosis and shorter overall survival time for lung adenocarcinoma patients by strata analysis. Finally, nuclear CCND1 expression tended to be an independent prognostic indicator (P=0.087) for lung adenocarcinoma patient survival. Conclusion: Increased nuclear CCND1 is a potential unfavorable prognostic factor for lung adenocarcinoma patients, especially those with clinical early stage (stage I+II).

Keywords: CCND1, lung adenocarcinoma, nuclear expression

Introduction

CCND1 belongs to the highly conserved cyclin family whose members are characterized by abundant expression throughout the cell cycle. CCND1 functions as regulator of CDK kinases and forms a complex with CDK4 or CDK6 promoting G1/S cell cycle transition. Overexpression of CCND1 is frequently observed in a variety of tumors including epithelial ovarian cancer, colorectal cancer, liver cancer, gastric cancer, nasopharyngeal carcinoma, and lung cancer resulting in altered cell cycle progression which contributes to tumorigenesis [1-6].

Lung adenocarcinoma, a histological subgroup in non-small cell lung cancer (NSCLC), is the most frequent form of lung cancer. In the US, nearly 40% of lung cancers are adenocarcinoma, which usually originate in peripheral lung tissue. Its incidence has been increasing in many developed Western nations in recent decades, where it has replaced squamous cell lung carcinoma to become the most common type of lung cancer in smokers as well as lifelong nonsmokers. In previous studies, CCND1 was observed to be overexpressed and attributed to the pathogenesis and poor prognosis of lung cancers [7, 8]. However, the expression pattern of CCND1 and its correlation with clinical features and prognosis in lung carcinoma have not been explored in detail.

In this study, we investigated CCND1 expression pattern in lung adenocarcinoma at both mRNA transcript and protein levels and evalu-

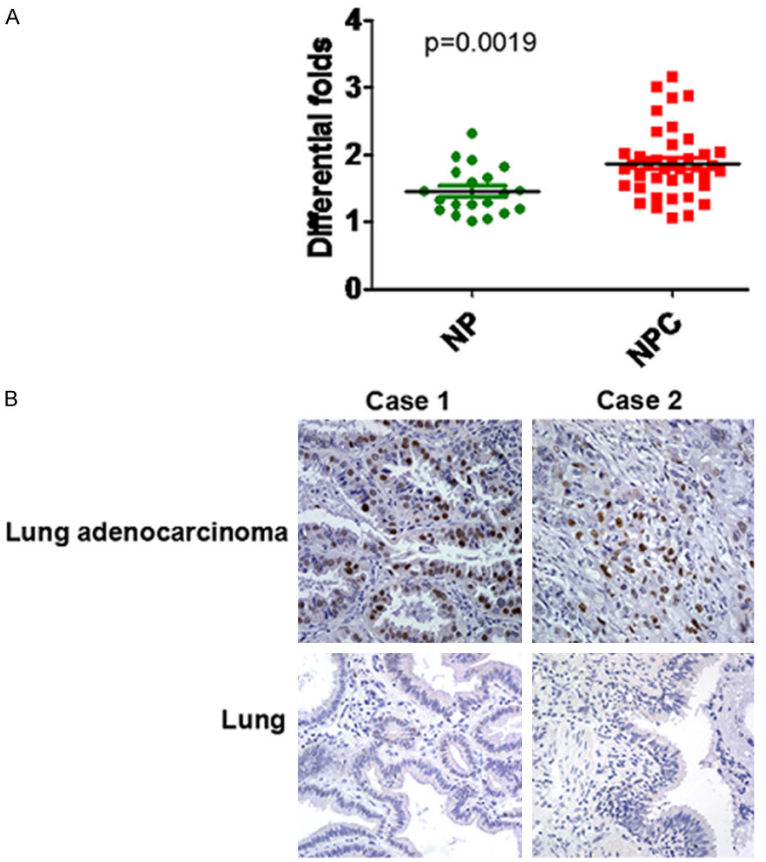

Figure 1. Upregulation of CCND1 in lung adenocarcinoma. A. Increased CCND1 mRNA was observed in lung adenocarcinoma compared to normal lung tissues. B. Increased CCND1 protein was predominantly expressed in lung adenocarcinoma tissues compared to lung tissues by immunohistochemistry.

ated its correlation with clinicopathologic features as well as patient survival. Our results confirm that CCND1 is an unfavorable prognostic factor promoting the pathogenesis of lung adenocarcinoma.

Materials and methods

Sample collection

Forty fresh lung adenocarcinoma tissues and 20 lung tissues were collected from the People's Hospital of Zhongshan City, China, at the time of diagnosis before therapy. All fresh samples were preserved immediately in liquid nitrogen. A tissue array including 75 paired paraffin-embedded lung adenocarcinoma and normal lung samples was were purchased from the National Engineering Center for BioChips in Shanghai, China. We also collected 72 paraffin-embedded lung adenocarcinoma samples from the People's Hospital of Zhongshan City. For the use of these clinical materials for research purposes, prior consent from the patients and approval from the Ethics Committee of this hospital were obtained. All specimens had confirmed pathological diagnosis and were staged according to the 2009 lung cancer staging system of the UICC.

Real-time polymerase chain reaction (PCR)

Real-time PCR was used to measure differential mRNA expression of CCND1 in 40 lung adenocarcinoma tissues and 20 lung tissues using a Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA) and SYBR Premix Ex Taq (Takara, Shiga, Japan), as described previously [9]. The sense primer was 5'-GCAGCAGAAGCGAGAGC-3', and the anti-sense primer was 5'-ACTTCTGTTCCTCGCAGAC-3'. The ACTB gene was amplified as an internal control using the sense primer 5'-TAAGGAGAAGCTGTGCTACG-3' and anti-sense primer 5'-GACTCGTCATACTCCTGCTT-3'.

Immunohistochemistry

Immumohistochemistry was performed as described [10] previously with a rabbit anti-human CCND1 polyclonal antibody at concentration of 1:100 (Santa Cruz Biotechnology, USA) (Santa Cruz Biotechnology, USA). Sections were visualized with DAB and counterstained with hematoxylin, mounted in neutral gum, and analyzed using a bright field microscope.

Nuclear protein extraction and Western blot analysis

Six paired lung adenocarcinoma and normal lung samples were frozen using liquid nitrogen and then respectively ground by hand to a fine powder with mortar and pestle. Subsequently,

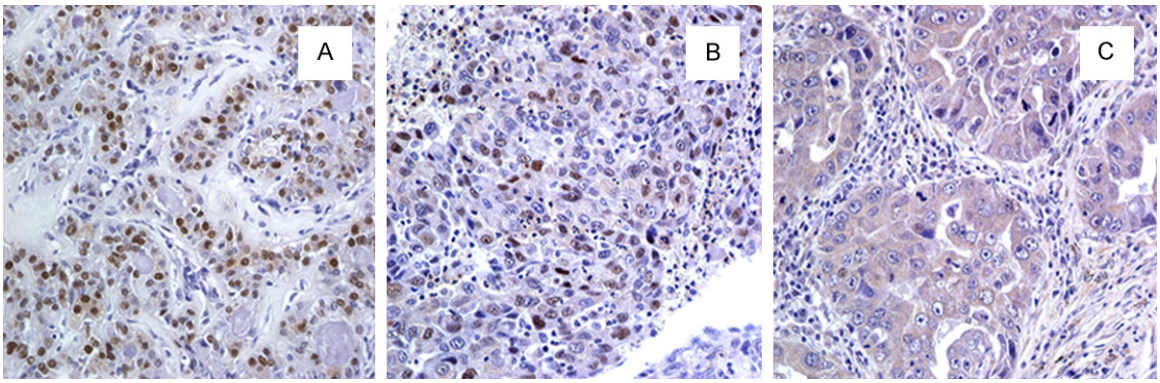

Figure 2. Different expression levels of CCND1 protein in lung adenocarcinoma tissues by immunohistochemistry.

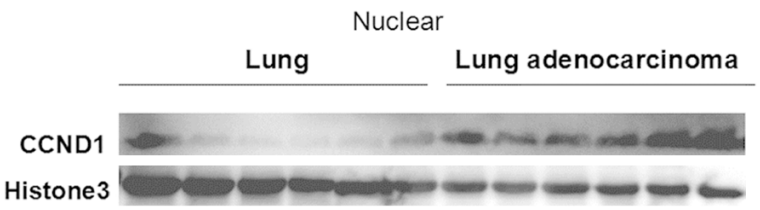

Figure 3. Increased CCND1 protein in lung adenocarcinoma tissues by western blot.

nuclear protein was extracted from these tissue powders according to the instruction of a nuclear protein extraction kit (Gaiji Inc, Nanjing, China). Protein lysates were resolved on 10% SDS polyacrylamide gel, electrotransferred to polyvinylidene fluoride membranes (Invitrogen, Inc. Carlsbad, CA, USA), and blocked in 5% non-fat dry milk in Tris-buffered saline, pH 7.5 (100 mM NaCl, 50 mM Tris and 0.1% Tween-20). Membranes were immunoblotted overnight at 4°C with a CCND1 antibody at a dilution of 1:400 (Abcam, 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 chemiluminescence (Pierce, Rockford, IL, USA).

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 more than or equal to 10% nuclear staining were considered as positive nuclear expression. Less than 10% staining was regarded as negative nuclear expression.

Statistical analyses

All statistical analyses were carried out using the spss software program (version 13.5; SPSS, Inc., Chicago, IL, USA). The t-test was used to analyze the differential expression of CCND1 mRNA between tumor tissues and normal tissues. The χ^2 test was utilized to analyze the relationship between CCND1 nuclear expression and clinicopathologic characteristics. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. The significances of various variables in survival were analyzed using multivariate Cox Proportional Hazards model. A *P* value of less than 0.05 was considered statistically significant.

Results

CCND1 mRNA is highly expressed in lung adenocarcinoma tissues

In order to understand the role of CCND1 in lung adenocarcinoma tissues, real-time PCR was used to measure the expression of CCND1 mRNA transcripts in 40 freshly collected lung adenocarcinoma tissues and 20 freshly collected normal lung tissues. Compared with healthy lung tissues, lung adenocarcinoma samples expressed significantly higher levels of CCND1 mRNA (P=0.0019) (**Figure 1**).

Increased nuclear protein expression of CCND1 in lung adenocarcinoma tissues

We next examined nuclear expression of CCND1 protein in 6 paired lung adenocarcino-

Table 1. The expression of CCND1 in lung adenocarcinoma (LA) and paracancerous tissue (PT)

Group	Cases (*n*)	Protein expression (*n*)		*P* value
		Low expression	High expression	
LA	140	68 (48.6%)	72 (51.4%)	P<0.001
PT	72	66 (89.3%)	6 (8.3%)	

Table 2. Correlation between the clinicopathological factors and expression of CCND1 in NSCLC specimens

Characteristics	n	CCND1 expression		
		High	Low	*P*
Age				
<60	66	38 (57.6%)	28 (42.4%)	0.126
≥60	74	33 (44.6%)	41 (55.4%)	
Gender				
Male	72	34 (47.2%)	41 (56.9%)	0.209
Female	68	38 (55.9%)	30 (44.1%)	
FIGO stage				
I+II	95	43 (45.3%)	52 (54.7%)	0.026
III	45	29 (64.4%)	16 (35.6%)	
Lymph node status				
Negative	75	40 (53.3%)	35 (46.7%)	0.376
Positive	65	32 (49.2%)	33 (50.8%)	
T classification				
T1+T2	119	64 (53.8%)	55 (46.2%)	0.138
T3+T4	21	8 (38.1%)	13 (61.9%)	
N classification				
N0+N1	100	49 (49.0%)	51 (51.0%)	0.235
N2+N3	40	23 (57.5%)	17 (42.5%)	
Distant metastasis				
M1	134	68 (50.7%)	66 (49.3%)	0.368
M0	6	4 (66.7%)	2 (33.3%)	

ma and lung tissues by western blot analysis. CCND1 nuclear expression was significantly increased in lung adenocarcinoma tissues compared to normal lung tissues (**Figure 2**).

Immunohistochemistry of CCND1 in lung adenocarcinoma and lung tissues

Expression levels CCND1 protein in 140 lung adenocarcinoma and 72 healthy lung tissues were examined by immunohistochemistry (**Figure 3**). We found that CCND1 protein staining was predominantly in the nuclei of tumor cells. Furthermore, we observed that 51.4% (72/140) (**Table 1**) cases showed positive nuclear CCND1 expression (**Figure 3**). However, in normal lung tissues, only 8.33% (6/72) cases positively stained for CCND1 (P<0.001).

Correlation between clinicopathological feature and CCND1 nuclear expression in NPC patients

The correlation between CCND1 nuclear expression and clinical characteristics was ‘also analyzed. As shown in **Table 2**, a significant correlation between nuclear CCND1 expression with patient’s age, sex, T classification, N classification, lymph node status or distant metastasis (M classification) in 140 lung adenocarcinoma cases was not observed. However, CCND1 expression was positively correlated with clinical stage (I-II vs. III) (P=0.026) in adenocarcinoma patients.

CCND1 nuclear expression negatively correlates with overall survival time of NPC

To assess the possible prognostic value of CCND1 expression for lung adenocarcinoma patients, Kaplan-Meier analysis with log-rank test was used. We observed that CCND1 levels were negatively correlated with overall survival time of lung adenocarcinoma patients. Patients whose tumors expressed CCNDI1 had worse prognoses than those considered negative expression (**Figure 4**) (P=0.019). Interestingly, we further observed that CCND1 expression in clinical stage I+II, but not clinical stage III, was associated with poor prognosis and led to the shorter overall survival times for lung adenocarcinoma patients by strata analysis.

Nuclear expression of CCND1 as an independent prognosis factor for lung adenocarcinoma patients

To investigate the potential of CCND1 nuclear expression as an independent prognosis marker, we used the Multivariate Cox Proportional Hazards model to analyze the significance of various variables in survival. Univariate analyses indicated that lymph node status, T, N, and CCND1 expression were significantly associated with patient survival (P=0.005, P=0.004, P<0.001, and P=0.022 respectively). Further, multivariate analyses adjusting for T classification and N classification of lung adenocarcinoma patients showed that CCND1 nuclear expression tended to be an independent prog-

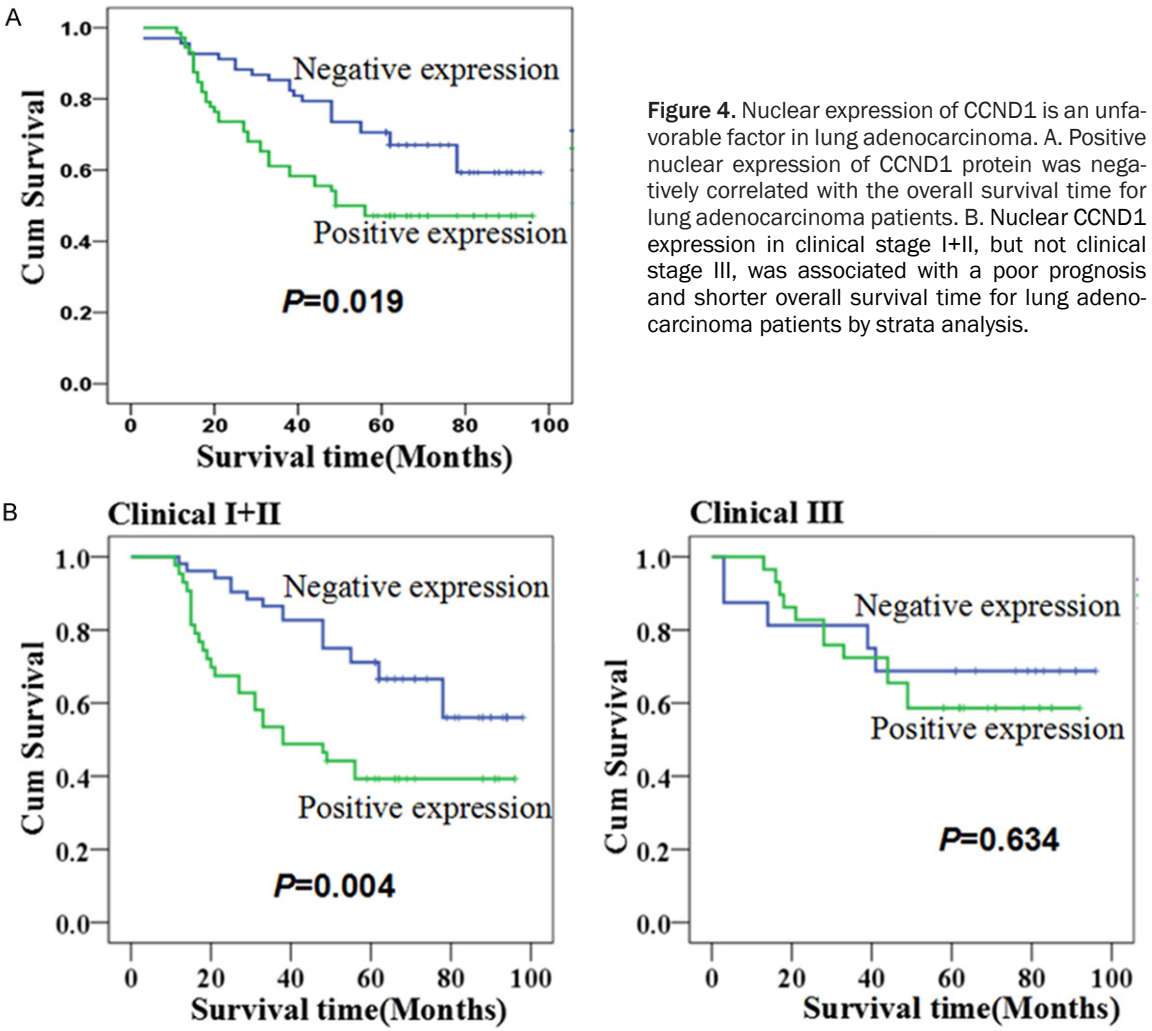

Figure 4. Nuclear expression of CCND1 is an unfavorable factor in lung adenocarcinoma. A. Positive nuclear expression of CCND1 protein was negatively correlated with the overall survival time for lung adenocarcinoma patients. B. Nuclear CCND1 expression in clinical stage I+II, but not clinical stage III, was associated with a poor prognosis and shorter overall survival time for lung adenocarcinoma patients by strata analysis.

nostic marker for lung adenocarcinoma patients (P=0.087) (**Table 3**).

Discussion

Cell cycle dysregulation is a common contributor to tumor proliferation in nearly all types of cancers. In this investigation, we focused on CCND1 expression to determine its potential role in lung adenocarcinoma. CCND1 has been reported to cooperate with CDK4 and CDK6 driving G1 to S cell cycle progression through the phosphorylation and subsequent inactivation of Rb protein. Abnormal CCND1 expression has been observed in a few tumors and promotes disease progression and poor prognoses in these patients. Nevertheless, the expression pattern of CCND1 and its correlation with clinical features or its prognosis value in lung adenocarcinoma were still unclear.

In this study, we observed that CCND1 mRNA expression was upregulated in lung adenocarcinoma tissues compared to healthy lung tissues. This finding was consistent with reports on laryngeal squamous cell carcinomas, neuroblastoma, and mantle cell lymphomas [11, 12], but in contrast to a study in human hepatocellular carcinoma [13]. The discrepancy between our data and Hu et al's data would be most likely due to the different tumor samples. Our study supports that CCND1 is involved in the pathogenesis of lung adenocarcinoma.

We observed that CCND1 expressed in both the nucleus and cytoplasm of lung adenocarcinoma and lung tissues by immunohistochemistry. Interestingly, only nuclear of CCND1 expression was specifically increased in lung adenocarcinoma specimens compared to lung tissues. This data was consistent with previous

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
Age						
<60 vs. ≥60	0.159	0. 694	0. 417-1.154			
Gender						
Male vs. Female	0.434	0.821	0.500-1.346			
Clinical stage						
I+II vs. II-III+III	0.342	1.309	0.751-2.284			
Lymph node status						
Negative vs. Positive	0.006	0.490	0.296-0.812	0.676	1.185	0.535-2.627
T classification						
T1+T2 vs. T3+T4	0.004	0.423	0.237-0.757	0.005	0.403	0.215-0.755
N classification						
N0+N1 vs. N2+N3	0.000	0.375	0.227-0.620	0.010	0.367	0.171-0.785
Distant metastasis						
M1 vs. M0	0.463	1.694	0.414-6.933			
CCND1 expression						
Low vs. high	0.022	0.553	0.333-0.917	0.087	0.629	0.370-1.070

investigations in bladder cancer, oral cavity squamous cell carcinoma, and colorectal carcinomas [14-16], which suggests that increased nuclear expression of CCND1 might stimulate the pathogenesis of lung adenocarcinoma. We also used western blot to confirm the high nuclear expression of CCND1 in lung adenocarcinoma samples compared to their respectively adjacent normal lung tissues.

In a previous study, CCND1 was reported to form active complexes with its binding partners cyclin dependent kinase 4 and 6 (CDK4 and CDK6) in the nucleus, which induces cell cycle progression [17] and promotes tumor cell growth. In this study, we demonstrated that although nuclear expression of CCND1 was not correlated with age, gender, T classification, N classification, M classification, it was positively related to clinical stage. Our data is similar with CCND1 reports in the context of prostate cancer [18], papillary thyroid carcinoma [19], colorectal cancer [20]. Together these studies provide strong support that nuclear expression of CCND1 promotes the progression of multiple tumor types, including lung adenocarcinoma.

Increased nuclear expression of CCND1 has been documented as an unfavorable factor in pancreatic cancer [21], gastric adenocarcinoma [22], and colorectal cancer [23]. Similar to these reports, we observed that nuclear CCND1 expression was markedly negatively correlated with the survival time of lung adenocarcinoma patients. Patients that exhibited positive nuclear CCND1 expression had an overall shorter survival time than those of patients who lacked CCND1 expression. Our data demonstrated the significance of nuclear CCND1 expression and further supported CCND1 as a key oncogene in lung adenocarcinoma. Interestingly, Mylona found that high nuclear CCND1 expression was a favorable factor for prolonging survival time in breast cancer patient subgroups with aggressive phenotypes [24]. This finding was inconsistent with our data, and may be attributed to the suppressive role of CCND1 in metastasis [25], which has not been investigated in the context of breast cancer. In further strata analysis against lung adenocarcinoma clinical stage, we observed that CCND1 expression in clinical stage I+II, but not clinical stage III was associated with poorer prognoses and shorter overall survival time. This suggests that CCND1 may be preferred as a biomarker for evaluating the prognosis of early and middle staged lung cancer patients.

In previous studies, CCND1 expression was a useful prognostic marker for some tumors [15, 22]. In this investigation, we confirmed that nuclear CCND1 expression is an independent prognostic factor for lung adenocarcinoma. Based on univariate analyses, patient's overall survival was inversely proportional to lymph node status, T/N classification, and CCND1 expression. Furthermore, CCND1 expression tended to be an independent prognostic marker of overall survival in multivariate analyses for NPC patients regardless of its patients' disease status.

In summary, our study indicates that CCND1 mRNA and protein levels are significantly elevated in lung adenocarcinoma tissues compared to normal lung. Furthermore, our data

suggests that nuclear expression of CCND1 is positively correlated with the clinical progression and is a poor prognostic factor for lung adenocarcinoma patients.

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Disclosure of conflict of interest

None.

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